A Thesis

entitled

Small Molecule Biomarkers Resulting from Radiation and Oxidative Damage to DNA

by

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Oxidative damage to DNA and other biomolecules is a result of various reactive radical species that are formed as a result of exogenous factors like radiation or by endogenous mechanisms involving cellular metabolic processes. The role of radiation in direct and indirect DNA damage is a fast growing field of study. Low-energy electrons (LEEs) are a fundamental component of Ionizing Radiation (IR) that are involved in various physicochemical and biochemical phenomena underlying radiation chemistry. This is based on the fact that exposure of biomolecules to such high energy radiation (particles and/or photons) causes electrons to be removed from any atomic or molecular orbitals of the matter accessible at that energy range. These liberated secondary electrons (SEs) can initiate further ionization or excitation processes thus leading to a cascade of events. Two types of radiation induced DNA damage have been studied, direct damage which refers to the direct energy deposition that inflicts damage leading to DNA strand breaks, and the indirect damage which refers to interaction with the molecular environment of DNA, chiefly water, liberating the reactive hydroxyl radical. It has been observed that the addition of LEE to DNA components, triggers the formation of unstable and reactive radicals and
ions. These reactive intermediates further generate carbon-centered radicals on the DNA nucleobase as well as the 2-deoxyribose moiety of DNA during the course of DNA damage. Though extensive study has established the importance of nucleobase damage, the oxidation on deoxyribose is also a critical aspect in the toxicology of oxidative stress. DNA damage pathway by LEE involves the cleavage of the phosphodiester bond leading to formation of the C3′-dideoxy and C5′-dideoxy radical at the C3′ and C5′ positions of deoxyribose. Site selective formation of such radical species through photolysis can be an effective means to study mechanisms involved in their formation and decide their metabolic fate. The goal of this research is to determine the fate of C5′-dideoxy radical by its site selective generation using photolysis. 5′-deoxy-C5′-thymidinyl radical precursor was synthesized using two different organic synthetic approaches and its suitability as a photochemical precursor for generating the 5′-deoxy-C5′-thymidinyl radical was evaluated. Fate of the radical formed can be investigated to develop potential biomarkers for oxidative stress which may in turn be significant towards quantifying the extent and pathogenesis of various diseases.

The secondary goal of this research is to synthesize and study the small molecules generated from 3′-oxonucleotide lesion. The base labile 3′-oxonucleotide lesion has been observed to undergo nucleobase elimination followed by further degradation to generate 2-methylene-3(2H)-furanone. To fully elucidate the impact of formation of these degradation products on DNA damage and repair processes, it is vital to understand the reactivity and chemical fate of these fragments. Attempts were made to synthesize these small molecule degradation products with focus on 2-methylene-3(2H)-furanone formation.
I dedicate this thesis to my lovely daughter Swara who gave birth to a ‘mother’
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List of Abbreviations

A………………….. Adenine
ACN……………….. Acetonitrile
AFM………………. Atomic Force Microscopy
Aq……………….. Aqueous

9-BBN…………… 9-borabicyclo (3.3.1)nonane
BH3……………….. Borane

C…………………... Cytosine
13C NMR………….. Carbon-13 Nuclear Magnetic Resonance Spectroscopy

dA………………… Deoxyadenosine
dC…………………. Deoxycytidine
DCM………………. Dichloromethane
DEA………………. Dissociative Electron Attachment
dG…………………. Deoxyguanosine
DMF……………….. N,N'-Dimethylformamide
DMSO…………….. Dimethyl Sulfoxide
DNA……………….. Deoxyribonucleic Acid
DSBs………………. Double Strand Breaks
dsDNA……………. Double Strand Deoxyribonucleic Acid

ESI-MS………….. Electrospray Ionization Mass Spectrometry

G………………….. Guanine

1H NMR………… Proton Nuclear Magnetic Resonance Spectroscopy
HPLC…………….. High Performance Liquid Chromatography

IR…………………. Ionizing Radiation

LEE……………….. Low-Energy Electron

NaHMDS……….. Sodium bis(trimethylsilyl)amide
NMR……………….. Nuclear Magnetic Resonance Spectroscopy
nm……………….. Nanometer
nM……………….. Nanomolar
8-oxodG.................8-Oxo-2’-deoxyguanosine
8-oxoG................8-Oxoguanine

ROS....................Reactive Oxygen Species
SEs.....................Secondary Electrons
SOPs....................Standard Operating Procedures
SSBs.....................Single Strand Breaks
ssDNA...................Single Strand Deoxyribonucleic Acid

T.........................Thymine
TBAF....................Tetrabutylammonium Fluoride
TBDMS...................tert-Butyldimethylsilyl
TBDMSCl...............tert-Butyldimethylsilyl Chloride
TEAA....................Triethylammonium Acetate Buffer
TEA......................Triethylamine
TFA......................Trifluoroacetic acid
THF......................Tetrahydrofuran
TNI......................Transient Negative Ion

UV.......................Ultraviolet
WHO.....................World Health Organization
List of Symbols

AB.........................Diatom molecule
Ar.........................Argon

CH₂Cl₂...............Dichloromethane
CHCl₃..............Chloroform
CH₃CN..............Acetonitrile
CH₃MgBr..........Methyl magnesium bromide
(C₂H₅)₂O.........Diethyl ether
CH₃P(C₆H₅)₃Br......Methyltriphenylphosphonium bromide
C₂H₅P(C₆H₅)₃Br.....Ethyltriphenylphosphonium bromide

ddT......................2',3'-Dideoxythymidine

E......................Energy
e......................Electron
e⁺(aq).................Solvated Electron
e⁺ enh..............Electron re-emitted into continuum
e⁺v ......................Electron transferred within DNA
eV..................Electron volts

GSH.................Glutathione reduced

HCl..................Hydrochloric acid
HF(aq)...........Hydrofluoric acid
H⁺..................Hydrogen atom
H⁺ aq./H₂O⁺.........Hydronium ion
H₂O⁺................Radical cation of water
H₂O* ..............Excited water molecule
HO₂⁺/ 'OOH..............Peroxyl radical

K₂CO₃ ...............Potassium carbonate
K⁺(CH₃)₃CO⁻........Potassium tert-butoxide
KMnO₄ ..............Potassium permanganate

MgSO₄ .................Magnesium sulfate

NaCl...................Sodium Chloride
NaHCO₃..................Sodium bicarbonate
NaOH………………..Sodium Hydroxide
Na₂SO₄………………Sodium sulfate

‘OH…………………..Hydroxyl radical
‘OH…………………..Hydroxyl ion
Chapter 1

Introduction and Background

1.1 Ionizing Radiation (IR)

As defined by the World Health Organization (WHO), ionizing radiation (IR) is radiation that has enough energy to remove the tightly bound electrons from the atomic or molecular orbital, thus causing ionization of the respective atom or molecule.\(^1\) The energy of waves from the Electromagnetic Spectrum, is directly proportional to its frequency while it varies inversely with its wave length. Hence, lower frequency waves have higher wavelength but less energy like microwaves and radio waves, and higher frequency waves have lower wavelength and higher energy like X-rays and gamma rays. Radiation waves from an electromagnetic spectrum can be ionizing and non-ionizing in nature. Ionizing radiation (IR) has adequate energy to release electrons from atoms or molecules. Since alpha particles and beta particles are charged particles, they are considered directly ionizing and can, therefore, interact with electrons in atomic shells through coulombic forces of attraction or repulsion. The neutron is a chargeless, indirectly ionizing particle. X-rays and gamma rays are electromagnetic in nature and constitute another type of indirectly ionizing radiation and they do not interact with
atomic electrons through electrostatic or coulombic forces. \[1\]

Ionizing radiation (IR) can originate from both natural and man-made sources. The extent of harm caused to humans depends upon the level of exposure and amount of energy imparted to or absorbed by the human body. Radiation is a ubiquitous part of the environment that always exists in the surroundings, be it in the air, food, water, soil, etc. A major proportion of the average annual exposure to radiation is a result of natural environmental sources, thus reflecting that it is a primary source of ionizing radiation.

Natural sources include cosmic rays which make their way through the atmosphere, there are radioactive decay products from various radioactive elements in earth’s crust, for example, radon gas which is generated from the decay of radium. Man-made sources mainly consist of medical diagnostics and therapeutics such as X-rays used in diagnostic radiology or the use of radiation in the treatment of disease like cancer, industrial sources includes byproducts from research, industrial waste, radioactive waste from nuclear stations. \[2\] The percentages of the average annual radiation exposure contributed by each of the above mentioned source is illustrated in Figure 1-1. \[3\] The total is about 360 millirems, almost 82 percent is from natural source; and only 18 percent is from industrial, medical, and other man-made sources.
Figure 1-1: Average annual exposure to Ionizing Radiation in U.S.A. [3]. Reprinted by permission.

Of all the various means of natural sources, radon gas is the largest source of radiation exposure to humans, its pathway of spreading is through air and soil. Medical exposure is considered to be the second largest source of IR, X-rays from diagnostics being the lead source. [3] Though IR can be used in the therapeutic treatment for diseases like cancer, it has also been proven to be a major element that can induce carcinogenesis. Therefore, IR can be considered to be a ‘double-edged sword’. IR mainly targets nuclear DNA and induces a series of reactions leading to the formation of a spectrum of damage products. DNA double-strand breaks (DSBs) induced by ionizing radiation are considered to be the most detrimental lesion as they result in genetic toxicity and carcinogenesis. In spite of cells having developed repair systems, the oxidative stress resulting from IR can interfere
with the repair mechanism leading to formation of unreppaired or mis-repaired DSBs which are a serious threat to genomic integrity. The most commonly discussed types of radiation emitted from natural or man-made sources, are alpha particles, beta particles, gamma rays, X-rays, cosmic radiation, and neutron radiation.

1.1.1 **Alpha particles (α)**

Alpha radiation is the flow of alpha particles that are released during radioactive decay, and each particle consists of two protons and two neutrons (equivalent to the helium-nucleus). The daughter nuclei formed has an atomic number by 2 less and an atomic mass number by 4 less as compared to the parent nuclei. Alpha particles carry a positive charge and are heavy, thus travel only short distances. Being charged, they interact strongly with matter. Alpha particles cannot penetrate topically, but if an alpha emitting substance is orally ingested via food or air, they cause serious cell damage. The ionizations they cause are clustered, therefore allowing them to release all their energy in a few cells. This results in lethal damage to cells and DNA.[4]

1.1.2 **Beta particles (β)**

Beta radiation consists of beta particles that are in the form of either an electron or a positron (a particle having the size and mass of an electron, but carries a positive charge) being emitted from a radioactive atom. Unlike alpha particles, beta particles have smaller masses, therefore, it is able to travel further in the air, up to a few meters. It has comparatively more penetrating power and can penetrate skin up to a few centimeters, posing a fair risk of an external health hazard. However, the primary threat still remains from internal emission caused from ingested material.[4]
1.1.3 Gamma rays (γ)

Gamma radiation, unlike alpha or beta radiation, is not made of particles, but is a flow of photons which are like bundles of energy coming from an unstable nucleus. As it does not possess any mass or charge, gamma radiation can travel much faster and farther through air in comparison with alpha or beta radiation. Gamma waves cannot be stopped by plastic or paper but can only be stopped by a thick or dense layered material, such as heavy metal plates; lead being the most effective form of shielding. Gamma rays have high penetrating potential and can easily penetrate barriers like clothing and skin and hence are a radiation hazard for the entire body. Gamma rays can easily pass through the human body and cause damage along their path. [4]

1.1.4 X-rays

X-rays are similar to gamma radiation in the context of being massless and uncharged. However, the primary difference is that, they originate from the electron cloud. X-rays consists of energy released during electronic transitions from within different atomic shells, like moving from a higher energy level to a lower one, thus releasing the excess energy. X-Rays have longer-wavelengths and slightly lower energies than gamma radiation, but its contribution to the DNA damage is substantial as well. Innumerable X-ray machines are used daily in diagnostics, and they are a major source of man-made radiation. [4]

1.1.5 Neutron Radiation (n)

Neutrons are chargeless and are usually emitted as a consequence of spontaneous
or induced nuclear fission. This radiation can travel over longer distance in the air (upto 1000 meters), but unlike gamma rays, neutrons do have mass and can be effectively blocked by a hydrogen-rich material such as water. Since neutrons lack charge, they cannot ionize an atom directly, but they can act as an indirectly ionizing agent. While they are absorbed into a stable atom, they make it unstable and more likely emit ionizing radiation of another type during the process. Its noteworthy that neutrons are the only form of radiation that is capable of turning stable nuclei into radioactive.^[4]

1.1.6 Cosmic rays

Cosmic rays are immensely energetic particles emerging from outside space around earth. They are a mixture of different types of radiation and are mainly composed of high energy protons and gaseous atomic nuclei which bombard the earth’s surface. They can have high energies, reaching above 1020 eV.^[4]

1.2 Interaction of IR with Biological Systems

High-energy ionizing radiation like α-rays, γ-rays, X-rays, etc., causes a high level of oxidative stress to living cells which generates numerous lesions, that can lead to mutation, loss of genetic information, biochemical instability of the cellular environment, and induce cell death. The biological damage caused by IR can be directly correlated to its potential to cause DNA damage. IR deposits energy discreetly along its path of propagation which determines the spatial distribution of lesions formed. Clustered DNA damage sites, can be a result of multiple lesions inflicted by ionizing radiation. The most commonly occurring and detrimental DNA lesions formed at the cellular level, which perturb cellular function, are those which affect human genome. These include single-strand breaks (SSBs), double-
strand breaks (DSBs), intra-stand and inter-stand DNA interactions, DNA-protein adducts or DNA-protein cross-links, formation of abasic sites and other biochemical modifications.[5][16]

These lesions could be a result of the immediate effect of high-energy quanta arising due to direct irradiation of the cell, or it could arise from secondary species, i.e., ions, radicals, excited atoms, and secondary electrons generated through the spontaneous loss of energy in femtoseconds. Thus, the resulting DNA damage is induced via two pathways; firstly, by the direct interaction of radiation with one of the DNA subunits causing electronic excitation, or secondly, by the indirect interaction with cellular components such as water, proteins, and salts surrounding the DNA to generate various reactive species. The indirect effect essentially arises from the IR-induced water radiolysis, which forms a series of products (hydroxyl radicals, hydrogen atoms and solvated electrons) that consequently damage DNA.[6][7] The base damage caused by IR has been extensively studied in vitro by site-specific irradiation of free bases, nucleosides, oligonucleotides or DNA itself in the solid state (dry) or in aqueous solutions (wet). Identification and analysis of radiation-induced DNA lesions is crucial in order to understand radiation-induced cell death, DNA strand breaks, biochemical changes within then cell and carcinogenesis, caused through initiation of genetic mutation and chromosomal aberration.[8]

1.2.1 Role of Secondary Electrons (SEs) or Low Energy Electrons (LEEs) in DNA damage

In both the direct and indirect effects, ‘ionization’ remains to be an integral part since the primary radiation loses its energy to the biological system mainly via ionization.
During this, large quantities of reactive species [e.g., ions, radicals, and large number of secondary electrons (SEs)] are generated along the radiation track. Typically, SEs are said to have initial kinetic energies lying below 30eV with a most probable energy of 9–10eV. When these electrons come across various molecules of the media, they lose their energies \( \textit{via} \) inelastic collisions. This transfer of energy can further initiate secondary excitations and ionization processes.\[9\]

SEs that possess energies below 30 eV are referred to as low-energy electrons (LEEs). When LEEs come across DNA components, they induce damage principally by attaching temporarily to these components, forming transient negative ions (TNIs) of DNA subunits (e.g., a base, deoxyribose sugar, or the phosphate group). These TNIs can then further dissociate or decay into electronically excited states and fragment \( \textit{via} \) dissociative electron attachment (DEA) channels that give rise to reactive species followed by SSBs and DSBs. This forms the pathway for LEE induced direct damage to DNA. Such direct LEE-induced DNA damage increases when the molecule is covalently bonded to radio sensitizers, chemotherapeutic agents and certain antibiotics. The indirect effect, deals with the interaction of radiation with the local biomolecules present around the DNA molecule, most importantly water and proteins. The water, on exposure to IR, can undergo radiolysis to create reactive species that can further form protein adducts to form unstable DNA damaged products. As a large proportion of the cell constituents (almost 70–80%) consist of water, the role of indirect damage by LEEs plays a substantial role.\[5],[10\]

Primary radiation consists of high-energy particles or fast charged particles like photoelectrons, which while passing across DNA molecules, cause rapid change of the
electromagnetic field along their path leading to DNA perturbation. These fast electrons can further cause excitation or ionization of other atoms in the cell and produce a large number of secondary LEEs. The energies of fast moving particles are used to overcome the nuclear binding energy of the electrons in the atomic shell. These electrons subsequently absorb the incoming energy and are ejected from an inner shell of an atom. Photon energy also provides kinetic energy to these ejected electrons. \[^{12}\] The probability of excitation and ionization is expected to be equal, but only about 20% of the energy deposited by fast moving charged particles in organic matter could lead to excitation, the remaining 80% may cause ionization. The ionization energy gets distributed in the form of potential energy of the cation obtained and the kinetic energy of secondary LEE formed. Consequently, the largest portion of the energy possessed by the primary particle is transferred in the form of the energy of the secondary electrons. \[^{10}\] Statistically, about 40% of the energy of the primary radiation is converted to that of secondary LEEs. This energy transfer from IR to DNA components occurs within femtoseconds of the instigating events, and the overall process can be divided into three groups: primary event, secondary event, and reactive event.\[^{11}\] These events have been illustrated in Figure 1-2\[^{11}\], where AB stands for a simple hypothetical diatomic molecule present within the cell. In the course of the primary events, the radiation deposited causes ionization and excitation of DNA components (reaction 1 and 2, Figure 1-2). During primary event, 80% of the energy absorbed by the system leads to reaction 1, which gives radical cations along with release of secondary electrons (SEs). This radical cation can further dissociate into fragment ions and more SE (reaction 4, Figure 1-2) or may undergo solvolysis to form nucleobase-OH adduct radicals, or may abstract protons from different sites of the sugar moiety, forming neutral C-centered
radicals. SE generated in primary events, i.e. **reactions 1 and 4**, have energies less than 30 eV, usually between 9 and 10 eV. These SEs are produced in large numbers (~ $3 \times 10^4$ MeV of deposited energy) and constitute a major portion of primary radiation energy.

![Diagram of ionization and excitation](image)

**Figure 1-2**: Ionization and excitation induced by primary ionizing radiation and secondary electrons.\(^{[11]}\)

The remaining 20% of the radiation energy absorbed can lead to molecular electronic excitation (**reaction 2, Figure 1-2**). The excited molecule can dissociate to produce neutral species (**reaction 5, Figure 1-2**) or ions (**reaction 6, Figure 1-2**). In the case where the excited molecule has enough energy for its own ionization, it may auto-ionize to generate
more SEs (reaction 3, Figure 1-2). The formation of secondary electrons is responsible for the secondary events shown in Figure 1-2. Secondary electrons interact with other molecules found in the cellular environment before their thermalization, and they induce more damage, which is referred to as the indirect effect of IR on biological systems. The different reactive species formed during primary and secondary events can lead to a chain of interactions with various biological molecules to generate new damage products, thus magnifying the effect. Therefore, LEE plays an indispensable role in IR-inflicted DNA damage. [12]

1.2.2 Mechanisms of the direct effects of Low Energy Electrons

The effect of LEE has been extensively studied and its role in inducing SSBs and DSBs in DNA has been well established. Considerable efforts have been made to explore the precise mechanisms responsible for localization of the lesions caused at a particular site and the overall damage inflicted. Atomic Force Microscopy (AFM) has proven to be useful to deduce the LEE-induced bond cleavage in a monomer. [5],[4] Another popular technique is X-ray photoelectronspectroscopy which is used to probe DNA damage and can give information about the bonding interactions and their effect on the DNA geometry. This technique involves adsorbing the DNA on thiolated gold substrates. [14] The results obtained from these different experiments have made it possible to predict the mechanisms responsible for bond cleavage in DNA. Two major types of Transient Negative Ions (TNIs) are believed to be involved in LEE-triggered DNA damage and these are formed on the basic subunits of the nucleoside. They are: shape resonances, in which the electron occupies a previously empty orbital present in the ground state of the subunit, and core-
excited or Feshbach resonances, in which the approaching electron is attracted and subsequently captured due to the high electron affinity of an electronically excited state of the subunit. If the energy of LEE is lower than the threshold for electronic excitation, TNIs are formed through shape resonances, whereas at higher energy, they were found to be a result of core-excited resonance.\textsuperscript{[5],[13],[14]}

Figure 1-3: Proposed routes leading to strand breaks and base release in DNA.\textsuperscript{[5]}

\[ e^{-} \] = electron re-emitted into continuum  
\[ e_{t}^{-} \] = Electron transferred within DNA  
DEA: Dissociative Electron Attachment.
Figure 1-3 represents the different decay channels followed by transient negative ions of DNA bases that initially exist at an electron energy of $E_0$. While the electron comes across the DNA, it is usually captured by a nucleobase, forming a shape or core-excited resonance depending on the imparted electron energy. The transient anion formed can then decay via three pathways: 1) the elastic ($E = E_0$) channel, used for electron transfer to the phosphate group or simply involves auto-ionization, 2) the direct dissociative electron attachment (DEA) channel, which is a major pathway leading to fragmentation accompanied by base release, and 3) the electronically inelastic ($E << E_0$) channel. As depicted in Figure 1-3, route 1 and route 3 involve transfer of electrons from the base subunit to the phosphate group where they can cleave the C–O bond of the DNA backbone at the 3′ and 5′ positions, to some extent, this cleavage can happen due to the direct capture of electrons at a phosphate group (energy less than 3eV). As mentioned earlier, core-excited types of resonances can lead to formation of TNI if the energy exceeds the electronic excitation threshold (i.e., above 3 eV), they can further decay via route 2, Dissociative Electron Attachment (DEA). Route 3 is the inelastic channel and involves release of a very low energy electron (e.g., $E_0$ between 0.5–1 eV) from the electronically excited base.\[5\],[15],[16]

1.2.3 Mechanisms of the indirect effects of Low Energy Electrons

Indirect damage occurs when primary radiation and SEs interacts with the system to produce reactive intermediates which then react with the local biomolecules present in the cellular environment that surrounds the DNA (e.g., water, salts, proteins, and oxygen). Since 70–80% of cell content is water, LEEs mainly react with water molecules near DNA and produces reactive species which subsequently lead to the indirect damage. The
The presence of water around DNA plays a critical role in determining the path of formation of TNIs, the decay channel of these TNIs, and the mechanism leading to the SSBs and DSBs. Water radiolysis by LEEs, ionizes H₂O to produce H⁻, ‘OH, H’ radicals along with solvated electrons. Formation of ‘OH radical can occur by DEA of the electronically excited states, LEEs with energies below 15 eV are captured by a water molecule around DNA to form a TNI, a core-excited Feshbach resonance, which can then decay via DEA. Recent theoretical studies suggest that the solvation of DNA molecules by polar molecules like water could significantly increase their ability to capture electrons, the solvated DNA bases are found to absorb electrons with near zero energies via the phenomenon of ‘adiabatic electron affinity’ modification.\[17],[18]

The initial ionization of water molecules by ionizing radiation is represented as,

\[
\text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^++ \text{SE (e)}
\]

It results in the formation of a water cation along with the formation of SEs. SEs generated here are rapidly transferred to the DNA subunits. Also the water cation can further interact with neighboring DNA molecules in the following manner,

\[
\text{SE(e)} + \text{DNA} \rightarrow \text{DNA}^- \quad \text{and}
\]

\[
\text{H}_2\text{O}^{++} \text{ DNA} \rightarrow \text{DNA}^- + \text{H}_2\text{O}^{++}.
\]

Since H₂O⁺ is acidic in nature, it also reacts with another water molecule, that is,

\[
\text{H}_2\text{O}^{++} + \text{H}_2\text{O} \rightarrow \text{H}_3\text{O}^+ + \text{’OH}
\]

The solvated electrons released may migrate over a distance by resonant electron transfer and interact with a series of neighboring water molecules.\[17\] The net reaction gives a ‘OH radical and a solvated electron (eₐq⁻), both of which are damage causing reactive species.
The contribution of SEs in the formation of 'OH is smaller than that produced via the ionization of water but it cannot be neglected. It is well established that the 'OH radicals formed as a result of water radiolysis increase the formation of SSBs and DSBs in DNA.\cite{17}

The elevation in DNA damage due to the addition of bulk-like water may happen via two mechanisms, (1) a rise in the number of reactive radical species like $\text{H}^-$, 'OH, and H’ in bulk water and (2) decrease in the kinetic energy of SEs, followed by their trapping into at the interface between the hydration water shell of DNA and bulk water molecules.\cite{18}

1.2.4 Theory of radiolysis of water

Water radiolysis occurs via three main stages taking place on different time scales are represented in Figure 1-4:

(1) The physical stage is achieved in about 1 fs, soon after ionizing radiation interacts with matter. It mainly consists of energy deposition followed by rapid relaxation processes. This stage involves formation of ionized water molecules ($\text H_2\text O^+$) along with the release of excited water molecules ($\text H_2\text O^*$) and formation of sub-excitations electrons ($\text e^-$).\cite{19}

(2) The physico-chemical stage, which occurs in the range of $10^{-15}$–$10^{-12}$ s, involves several processes including; (i) ion-molecule and ion-radical interaction, (ii) auto-ionization of excited states, and (iii) solvation of sub-excitation electrons.

(3) The chemical stage, which is attained in $10^{-12}$–$10^{-6}$s, involves the reaction of species along the ionization track that diffuses through the solution. Thus, in this stage, the intermediates formed react amongst each other as well as with surrounding biomolecules. The spectrum of the products widens due to diffusion of radicals and their succeeding chemical reactions.
Knowledge of the radiolysis products of water molecules can help in interpreting various damaged products of DNA.

Figure 1-4: Three stages in water radiolysis [19] Reprinted with permission.
1.3 DNA Damage by Hydroxyl Radicals (’OH)

1.3.1 Base Damage in DNA by Hydroxyl Radicals (’OH)

Hydroxyl radicals play an important role in free-radical induced oxidative DNA damage. These radicals can be generated exogenously by the radiolysis of water by IR, xenobiotics, environmental pollutants or generated endogenously via various cellular metabolic processes, or in diseases which trigger the immune system like chronic infection and inflammation. The hydroxyl radical is extremely reactive and can easily diffuse through the cell. It reacts with biomolecules such as DNA, proteins, lipids, etc., causing chemical modifications. The electrophilic nature of this radical can contribute to the damage caused to the nucleobase and the sugar moiety of DNA. With respect to nucleobase, ’OH can add to double bonds of the DNA bases or abstract a H atom. It can add to the C-C double bonds of pyrimidines or the C-C and C-N double bonds of purines in DNA. The addition of the ’OH to the double bonds of nucleobases is selective, depending upon the difference in electronegativity of different C-atoms in the base. For example, it will favor addition to C5 of thymine which is more electron rich over the less electron rich C6 position. [9] [20] Hydroxyl radical on addition to the C5-C6 double bond of pyrimidines bases like adenine and guanine, leads to formation of C5-OH and C6-OH adduct radicals respectively. ’OH can also abstract H atoms from methyl thymine resulting in the generation of an allyl radical at that position. Redox properties of these adduct radicals are different and play a role in determining the further reactivity of the respective radical. For examples, the C5-OH adduct radical is reducing in nature therefore is more easily oxidized than the C6-OH adduct radical that is oxidizing. [20] Therefore, radicals fate depends upon
their redox properties and the surrounding environment. Pyrimidine radicals give rise to numerous products, formation of these products is highly influenced by the absence or presence of oxygen. For instance, in the absence of oxygen, the C5-OH adduct radical of pyrimidine bases undergoes addition of water followed by deprotonation, leading to formation of cytosine glycol and thymine glycol. C5-OH-peroxyl radicals are generated by the addition of oxygen to C5-OH adduct radicals at diffusion-controlled rates. C5-OH-peroxyl radicals can spontaneously eliminate O$_2^+$, followed by reaction with water to give thymine and cytosine glycols.$^{[21][22]}$ The C6-OH adduct radical can undergo a similar fate. Apart from adding to the double bonds in nucleobases, the 'OH can also abstract hydrogen atoms from the methyl group of thymine, as well as generate base adducts through addition to the C4, C5, and C8 positions of purines. The allyl radical resulting from H-abstraction has no oxidizing or reducing properties. The C4-OH and C5-OH adduct radicals of purines lose water and are converted to strongly oxidizing purine (-H) • radicals, which may undergo one-electron reduction and are protonated to reconstitute the purine. In the case of adenine, C4-OH and C8-OH adducts are the two commonly formed OH adduct radicals. C4-OH adduct radicals have oxidizing properties, whereas C8-OH adduct radicals are mainly reducing in nature. $^{[21]}$

1.3.2 Sugar Damage in DNA by Hydroxyl Radicals ('OH)

Although the chemistry of nucleobase damage has been extensively studied and documented, the role that oxidation of deoxyribose in DNA plays in radical induced damage, oxidative stress and resultant genetic toxicity cannot be neglected. There is growing evidence that radical-mediated oxidation of deoxyribose in DNA generates
numerous diffusible electrophiles that can take part in various reactions with surrounding biomolecules, forming covalently bound fragments and oxidized basic sites. Oxidation of each of the five positions in 2-deoxyribose in DNA gives rise to a unique spectrum of products specific for a given carbon.

Figure 1-5: Formation of various C-centered radicals in 2-deoxyribose. [25]
Hydroxyl radicals being extremely reactive, abstract hydrogen atoms from the five carbon positions of the 2-deoxyribose moiety of DNA, leading to five C-centered radicals as shown in Figure 1-5. Damage to 2-deoxyribose in DNA leads to strand breaks and abasic site formation along with the release of unaltered DNA base products. The C-centered radicals undergo further interactions, giving rise to a variety of 2-deoxyribose products, some of which are released from DNA, while the remaining stay within DNA in the form of end groups of broken DNA strands.

The extent of 'OH attack on 2-deoxyribose in DNA generally amounts to less than 20%, although this percentage may vary in the cell nucleus. However, the DNA strand breaks observed are greater than expected from the amount of 'OH attack on 2-deoxyribose, which indicates a possible radical transfer from the nucleobase radical to the 2-deoxyribose sugar. The order of reactivity of 'OH toward each hydrogen atom of the 2-deoxyribose moiety depends upon solvent accessibility of the sugar hydrogen atoms in B-form DNA. These findings showed that 'OH abstracts a H' from the five carbons in the order H5' > H4' > H3' ≈ H2' ≈ H1′ and that the C4' - and the C5' -positions are the most accessible to solvent from the minor groove. The chemistry of each of these sugar radicals is discussed in the following sections. Though the energies required for H' abstractions from the different positions of the 2-deoxyribose by 'OH can be correlated directly with the strength of the C–H bonds, the solvent accessibility of these structures in surrounding solvent plays a critical role for 2-deoxyribose situated within DNA. [9] Easy accessibility of H4’ and H5’ to H’ abstraction by 'OH can be attributed to its exposure to solvent. The
accessibility of H1′ is very low in the B-form of DNA. The C4′ radical, due to its easy accessibility, appears to be the major radical produced by H′ abstraction from 2-deoxyribose is in DNA. Heterolytic cleavage of the phosphate group at C3′ and C5′ leads to strand breakage and formation of radical cations. In presence of oxygen, hydration of these C-centered radicals can give rise to unaltered base release along with formation of small molecular end groups will be discussed later in this section. [23][24][25]

1.3.3 Fate of C1′-radical

Various nucleic acid damaging agents can abstract the anomeric hydrogen of nucleotides. Amongst them are oxidants capable of forming the 2-deoxyribonolactone abasic site including copper–phenanthroline complexes, cationic manganese porphyrins, oxoruthenium complexes, UV irradiation, enediyne antibiotics, and γ-radiation. In a B-DNA helix the position of H1′ is buried in the minor groove which makes it relatively inaccessible to solvent. The Cl′-position of nucleotides plays a role in a variety of nucleic acid damage processes. Reaction at this C-center can result in premutagenic α-deoxyribonucleotides, alkaline-labile lesions, or direct strand breaks.[26] The C1′-radical may be generated through different mechanisms like hydrogen atom abstraction from the native nucleotide by species such as the hydroxyl radical, deprotonation from the C1′-position by the nucleobase radical cation or by internucleotidyl hydrogen atom abstraction. α-Deoxynucleotides are potential DNA damaging lesions that may be formed under oxidative stress conditions where the C1′-hydrogen atom is reduced by a thiol like GSH. It is challenging to measure the efficiency by which the initially formed C1′-radical is converted into the α -anomer of the respective nucleotide. Furthermore, the efficiency of
α-deoxynucleotide formation must also be discussed in the context of other existing competing reactions, for instance, quenching of the radical precursor by O₂ which leads to the formation of 2-deoxyribonolactone lesion.

Extensive study has been carried out by Sigman and his group [27] to investigate the abstraction of the Cl′-hydrogen from nucleotides in a duplex DNA by the neocarzinostatin chromophore, esperamicin A and bis(1,l0-phenanthroline)copper, resulting in the formation of the 2-deoxyribonolactone which is an alkaline labile lesion. Nevertheless, only bis(1,l0-phenanthroline)copper is believed to result in direct strand breaks via a Cl′-deoxyribonucleotide radical intermediate. This is observed to be one of the best-characterized DNA cleavage reactions. γ-Radiolysis can give the product resulting from Cl′-hydrogen atom abstraction through a two-step process involving, firstly, nucleobase ionization, followed by deprotonation from the anomeric carbon. Greenberg’s group was successful in synthesizing a radical precursor, that on photolysis generated a C1′-uridinyl radical at a defined site in single and double-stranded oligonucleotides. Often anomeric nucleotide radicals like 2′-deoxyuridin-1′-yl (7) are believed to be a part of DNA-damage processes. [26] The C1′-uridinyl radical formed can undergo different interactions under aerobic and anaerobic conditions as illustrated in Scheme 1-1. When the radical is trapped by O₂, the C1′ lesion is ‘fixed’ and results in the formation of either a direct strand break, or an alkaline labile lesion. [26] [27] Furthermore, independent generation of deoxyuridin-1′-yl radical 10 makes it possible to determine the competition between O₂ and a thiol for the radical. These investigations helped to prove the dependence of direct strand breaks and alkaline labile lesions with thiol concentration. [28]
Under aerobic conditions, radical 7 is trapped by oxygen at a diffusion-controlled rate to generate peroxyl radical 11, which is reduced by the thiol group of GSH to produce ribonolactone 9 along with release of the base 8. In absence of the thiol, the peroxyl radical 11 releases superoxide to generate a carbocation 12, which on exposure to nucleophilic attack by water gives ribonolactone 9.\(^\text{[28]}\) Though the ribonolactone is more stable compared to a native abasic site (\(~10–50\)-fold), it undergoes a rate-limiting β-elimination reaction to give a butenolide species 14 (half-life of 20 hr in single-stranded DNA and about 32–54 hr in duplex DNA), which undergoes rapid δ-elimination to give 5-methylene-2(5H)-furanone 16.\(^\text{[29]}\)\(^\text{[30]}\) 16, being strongly electrophilic, can react with nucleophilic components present in the cell. Independent generation of deoxyuridin-1′-yl radical 7 using photolysis, under anaerobic conditions, establishes that any α,β-deoxyuridine 10a, 10b found in a biopolymer must be derived from radical 7. Subsequently, the stereoselectivity of the reduction of the C1′-radical can be determined. The results from these various studies can be effectively used to determine the fate of the C1′-radical and offer a basis to study the reactivity of important reactive intermediates formed.
Scheme 1-1: Fate of the 2'-deoxyuridin-1'-yl radical. [31]
1.3.4 Fate of C2′-radical

To date, the 2′-position of deoxyribose is not a commonly investigated abstraction site in DNA, most likely due to its low solvent accessibility or low reactivity of the hydrogens at this position to abstraction. Isao Saito and his co-workers observed the oxidized abasic site that is formed as a result of formal hydrogen atom abstraction from the C2′-nucleotide position during γ-irradiation of DNA as well as photolysis of DNA comprised of 5-halopyrimidines [27]. C2′ hydrogen atoms in DNA undergo abstraction under aerobic conditions to produce an oxidized abasic lesion (C2-AP, 20), along with other products of DNA damage as represented in Scheme 1-2. C2-AP is also termed as erythrose abasic site, the effects of C2-AP on DNA structure and function are not yet clear. Greenberg and his group have successfully synthesized oligonucleotides containing this lesion. [32] Oxidation of the 2′-position during γ-irradiation and by photoinduced generation of a 2′-radical in a 5-iodouracil-containing oligodeoxynucleotide has been studied. [27][32]

The incorporation of 5-halouracils into DNA can increase the photosensitivity with respect to DNA-protein-crosslinking, DNA strand breaks, and the formation of alkali-labile sites. Additionally, uracil is known to form radicals that act as efficient hydrogen abstractors and are capable of abstracting hydrogen atoms from adjacent sugars moieties. Recent results suggest that certain chemical modifications of DNA like oligonucleotides containing a halogenated uracil at one site that is cleaved by photooxidation can give products that can be accounted for by a mechanism involving 2′-H abstraction. [33][34] Noticeably, the erythrose abasic site (20) is significantly more stable to hydrolysis than the native and other oxidized abasic sites, with a half-life of 3h in 0.1 M NaOH at 37 °C. The conformation of
DNA plays a major role in deciding the fate of 18. For instance, when 18 is produced in B-form of DNA, the erythrose abasic site is the key product, while in Z-form DNA the primary product is found to be ribonucleotide.

Scheme 1-2: Fate of C2'-deoxyuridinyl radical generated from γ-irradiation of poly(U)

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Oxygen reacts with the 2'-deoxyribosyl radical 18 to generate a peroxyl radical 19 that may homolytically or heterolytically cleave to form the erythrose-containing site 20. One chemically remarkable, but biologically irrelevant, feature of this abasic site is that, when heated under alkaline conditions, it leads to a retroaldol reaction resulting in a strand break and forming phosphoglycaldehyde fragmented termini, both the 3’ end and the 5’ end (22 and 23) as represented in Scheme 1-2. These termini have been detected and quantified by HPLC. [27] [32]
1.3.5 Fate of C3′-radical

The hydrogen atom at C3′, situated in the major groove, appears to be involved in several pathways that have been extensively investigated. The majority of known oxidative cleavage agents bind in the minor groove rather than the major groove, thus making the study of those formed from C3′ position uncommon. Therefore, oxidation of the 3′-position in DNA presents a more complicated case than that of 2′-oxidation. Bryant-Friedrich and her group have extensively studied the chemistry at C3′ position at monomeric levels, as well in oligonucleotides. The significant products obtained under aerobic condition are those fragments that contain a 3′-phosphate (3′-P), 3′-phosphoglycolate (3′-PG), and 3′-phosphoglycolaldehyde (3′-PGA). While 3′-phosphate-terminated fragments are produced in different amounts under aerobic conditions and can result from hydrogen atom abstraction that takes place from any position of the 2′-deoxyribose moiety, 3′-PG is formed additionally upon 4′-hydrogen atom abstraction, and 3′-PGA is formed as a result of 2′ and 3′-hydrogen atom abstraction. The fact that many of these products are electrophilic in nature and their formation occurs through a single oxidative step can make the presence of a C3′-DNA radical more detrimental to the cell as compared to other frequently occurring C-centered radicals.\cite{35,36} When DNA is exposed to ionizing radiation, 3′-PGs are found to be a significant damage fragment accounting for up to 50% of the modifications found at strand break sites and 100% of sites in the case of bleomycin-induced strand scission. 3′-PGA (22) has been detected during photocleavage of DNA under aerobic conditions induced by rhodium complexes that slightly unwind the DNA.
The fate of the C3′ radical under aerobic conditions has been studied by researchers like Dedon and his group at MIT. This aerobic pathway is depicted in Scheme 1-3. Amongst the major products observed in this case are; a DNA strand with a 3′-phosphoglycaldehyde termini group 22, base propenoate 28, along with a 5′-phosphate-terminated DNA strand 13. During this, the C3′-radical is attacked by oxygen to form a peroxyl intermediate 24, which can rearrange to give oxygen atom insertion into the deoxyribose ring, forming cationic radical 25 that reacts with water and decomposes to yield DNA fragments with 5′-phosphate (13) or 3′phosphoglycaldehyde termini (22). Oxygen was found to be essential for formation of the 3′-phosphoglycaldehyde-terminal fragment and base propenoate, but not for formation of free base or the 3′-phosphate-terminated product.

Scheme 1-3: Proposed H-3′-Abstraction Pathway under Aerobic Conditions. Reprinted with permission.
The anaerobic pathway (Scheme 1-4) involves oxidation of the 3'-radical, followed by solvolysis by H$_2$O to give alcohol 29 that then undergoes β-elimination to generate 3'- as well 5'-phosphate termini (15 and 13 respectively) accompanied with the release of free base,32. This mechanism involves the formation of an unopened lactone derivative, 2-methylene-3(2H)-furanone (31), formed via β-/δ-elimination of the 3'-oxo-nucleotide residue. As of now, there is limited literature supporting that undergoes to release 2-methylene-3(2H)-furanone (31), though its formation has been observed.$^{[38]}$

Scheme 1-4: Proposed H-3'-Abstraction Pathway under Anaerobic Conditions. $^{[27]}

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On further advances in this work, it was found that C3'-radical has been successfully generated in 2-deoxyribose moiety at a single molecule level as well as in modified oligonucleotides. $^{[39]}$ The broad spectrum of products observed from C3'-thymidinyl
radical degradation in DNA can be attributed to the flexibility of the ssDNA oligomer that allows easy relocation of the reactive radical intermediates within a DNA strand via hydrogen atom abstraction or resonant electron transfer process.\textsuperscript{[39] [40], [41]}

The fragments obtained from C3′-radical (4) clearly indicates the preference for intramolecular hydrogen atom abstraction. The C3′-radical is a prochiral site and is therefore accessible to oxygen from both directions, the top as well the bottom faces of the molecule. The peroxyl radical 24, initially formed from the C3′-radical can justify the formation of the 5′-aldehyde at the nucleoside along with the 4′-oxidation product and 3′-PG that can be explained by the formation of a C5′ and a C4′-radical in the oligonucleotide strand. This phenomenon has been outlined in Scheme 1-5. The 3′-peroxyl radical (24) lies in close proximity of the 4′-hydrogen of the same nucleotide and the 5′-hydrogen of the 3′-adjacent nucleotide. These studies display intramolecular abstraction of these hydrogens by the 3′-peroxyl radical 24 could result in the formation of both the 3′-PGA and the 5′-aldehyde detected through radical intermediates 32 and 33 respectively (Scheme 1-5)\textsuperscript{[38]}

 Scheme 1-5: Intramolecular Hydrogen Atom Abstraction by the C3′-Peroxyl radical \textsuperscript{[38]}

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1.3.6 Fate of C4′-radical

C4′ radical induced damage, and the resulting strand scission, has been extensively observed in many systems. B. Giese and his group have extensively studied the chemistry of single-stranded C4′-DNA radicals under anaerobic and aerobic conditions.[40] This position has been investigated in detail because it is a solvent accessible position in deoxyribose in B-form DNA. Therefore, it is expected that many DNA-interacting molecules react at this site. Amongst the oxidative damage studied at the five different carbons of the 2-deoxyribose, oxidation of the C4′-position has been the most exhaustively studied and various models for C4′ radical generation are available. Ionizing radiation, EDTA-iron complexes, hydroxyl radicals, and drugs, mainly antibiotics like bleomycin and elsamicin A, can participate in DNA damage initiated from 4′-hydrogen atom abstraction. One of the significant studies with respect to this involved the study of the antitumor antibiotic, bleomycin, that was found to cause 4′-oxidation and generate single- and double-strand DNA lesions. The H-4′ radical can be termed a β-phosphatoxyalkyl radical which has two phosphate groups beta to the radical, hence making the phosphate elimination easy and quick. The chemistry of 4′-oxidation proceeds along either of the two possible pathways to give various stable and electrophilic products. One pathway involves formation of oxidized abasic site comprised of 2-deoxypentos-4-ULOSE residue 36 as represented in Scheme 1-6. The other pathway has been recently studied and can proceed along distinct reaction pathways depending on the oxidizing agent. [38] The nature of the products formed depends upon the damaging agent involved. For example, a base propenal is formed from bleomycin and enediyne antibiotics, and free nucleobase or malondialdehyde has been observed for EDTA-iron complexes. [27] [41] A mechanism for
4'-hydrogen abstraction was first proposed during the γ-radiation induced DNA strand breaks in an attempt to explain the formation of scission fragments having phosphate termini along with ketone sugars. **Scheme 1-6** below represents anaerobic pathway.

Scheme 1-6: Proposed H-4'-Abstraction Pathway mediated by Gamma radiolysis under Anaerobic Conditions. [27]

The C4' radical 5 is converted into radical cation intermediate 34, which, being unstable, will disintegrate to give open-ring sugar molecule 35, 3’ phosphate termini 15, along with base release. Under anaerobic conditions, the C4'-deoxyribosyl radical 5 in ss- or ds-DNA undergoes heterolytic cleavage to generate radical cation 37 which on hydrolysis, fragments to give radical 38 and a 5’-phosphate termini 13 or can generate radical 40 that can subsequently undergo β-fragmentation. Thus, a C4’-radical can cause the molecule to
undergo β-elimination of one or both phosphates. A different mechanism was postulated for gamma radiolysis to study the products produced under aerobic conditions (Scheme 1-7). In this pathway oxygen reacts at a diffusion-controlled rate at the C4′-radical center to produce a peroxyl radical. A Russell-type mechanism implicates the combination of two such peroxyl radicals to form tetraoxide, which can further undergo homolytic cleavage to give oxyl radical, which undergoes β-fragmentation and reacts with oxygen to yield open ring compounds and base propanal. Fragmentation of these intermediates can release a 5′-phosphate terminal fragment, 3′-phosphoglycolate terminal product and base propanal.

Scheme 1-7: Proposed H-4′-Abstraction Pathway mediated by Gamma radiolysis under Aerobic Conditions Reprinted by permission
The homolytic bond scission gives rise to the 2-deoxyribose sugar radical that can subsequently undergo radical recombination or secondary oxidation that can actually interfere with the succeeding chemistry of the radical. Evidence also suggests the formation of DNA adducts with one of the products of 4′-oxidation of DNA. The base propenal species (49) has been proven to be an important though not major source of the endogenous pyrimidopurinone adduct of M1dG and dG accompanying the formation of 3′-phosphoglycolate residues by various oxidants (e.g., bleomycin, and enediyynes). \[^{38,44}\]

**1.3.7 Fate of C5′-radical**

Like the 4′ hydrogen, the two hydrogen atoms at the 5′-carbon of 2-deoxyribose are also highly accessible in a B-DNA helix. DNA scission mediated by abstraction of a hydrogen from the 5′-position has been predicted for various damaging agents like the enediyne antibiotics, hydroxyl radicals, gamma radiation, and metal cations like porphyrins. 5′-oxidation chemistry branches along either of two pathways (Scheme 1-8). One path yields a strand break having 3′-formylphosphate 50 termini and 5′-(2-phosphoryl-1,4-dioxo-2-butane) 51 as terminal fragments, whereas the other results in a strand break forming a 3′-phosphate and nucleoside-5′-aldehyde residue 52. \[^{38}\]

![Scheme 1-8: Two pathways of 5′C-oxidation \[^{38}\]](image_url)
In spite of the easy accessibility of the 5′C site, the chemistry of the 5′-oxidation of 2-deoxyribose in DNA has not received the same consideration as the 1′ and 4′ positions with regards to models designed for 5′ radical generation and biological studies. However, 5′-oxidation leads to the formation of highly electrophilic products that have generated interest with respect to their biological consequences.

Scheme 1-9: Formation of Furfural 54 from 5′-aldehyde lesion. [38]

5′-nucleoside-5′-aldehyde residue 52 undergoes δ-elimination reactions to release furfural (54) as depicted in Scheme 1-9, the physicochemical properties of which are on the lines of those of the methyl furanone species generated from δ elimination reactions in the case of the 3′-oxonucleotide and the 2-deoxyribonolactone products that were described in the previous section. The 5′ position of purine nucleosides undergo hydrogen abstraction forming 5′-8-cyclonucleosides, which have been perceived as one of the decomposition products of DNA due to exposure to ionizing radiations or upon chemical treatment by highly oxidizing radical species. [45] As shown in Scheme 1-10, under aerobic condition, 5′-deoxyribosyl radical reacts with oxygen to form 5′-peroxyl radical 55, which is predicted to be trapped by thiol, and the subsequent chemistry yields a DNA strand with 5′-aldehyde group 52 and a DNA strand terminated with a 3′-phosphate group, 15. Aldehyde product
52 is distinctive to agents that oxidize the C5’-position and can therefore serve as a biomarker for the C5’ damage lesion.\textsuperscript{[27]}

Scheme 1-10: Fate of C5 ’-deoxyribosyl radical.\textsuperscript{[27],[38]}

5’-(2-Phosphoryl-1,4-dioxobutane) in its cyclic form (57) can exist in equilibrium with the open chain form (51), this dialdehydic lesion further undergoes β-elimination to yield a highly reactive α,β unsaturated dicarbonyl species.\textsuperscript{[38],[45]}

Under anaerobic conditions, the chemical fate of the C5’ radical is unclear. All proposed intermediates proposed are rationalized by the products observed in DNA degradation due to neocarzinostatin (NCS) chromophore in the presence of GSH where 6 is trapped by GSH resulting in formation of 1. It was displayed that furfural can form adducts with adenine residues in DNA. This adduct successively undergoes deglycosylation to release kinetin 59 and abasic site 60 (Scheme 1-11).\textsuperscript{[46]}
Scheme 1-11: Adduct formation between furfural and an adenine residue in DNA. [46]

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1.4 Generation of 2-methylene-3(2H)-furanone

The 2-deoxyribonolactone lesion resulting from oxidation of the C1’ position of DNA nucleotides is a commonly occuring abasic site that can lead to DNA strand breaks, mutations and DNA-protein cross-links. 2-deoxyribonolactone, is a result of various DNA damage causing agents like γ-radiolysis, antitumor antibiotics, copper–phenanthroline complexes. This lesion has been successfully generated using photolysis at a specific site of an oligonucleotides under aerobic conditions and it has been effectively characterized by electrospray mass spectrometry (ESI-MS) and gel electrophoresis. As explained in Scheme 1-1, oxidation of the C1’ radical of deoxyribose in DNA gives rise to the 2-Deoxyribonolactone (or oxidized abasic site) lesion 9 within DNA. 2’-deoxyribonolactone is fairly unstable and can readily undergo β- and γ-elimination reactions with the release of the 3’- and 5’-phosphonated fragments, respectively. This leads to DNA strand scission along with release of 5-methylene-2-furanone, 16 [47],[48] that can be isolated by subsequent treatment of reaction mixture with β-mercaptoethanol which gives thiol adduct of 16. An analogous β/δ- elimination reaction with the 3’-oxonucleotide product of 3’-oxidative
damage yields 2-methylene-3(2H)-furanone (5-MF,31) as seen in Scheme 1-12. Its properties could be predicted on similar lines as that of 5-MF. As of now, no formal method has been devised for the study of its chemical reactivity.

**Scheme 1-12:** Proposed formation of 2-methylene-3(2H)-furanone from 3'-oxo-lesion. [46]

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A significant contribution of work involving this reactive unsaturated species generated by the abstraction of a C3'-hydrogen from a deoxyribonucleotide is the investigation of photonucleases designed by Barton and co-workers. Upon activation with light, these Rh-phenanthrenequinone diimine complexes abstract the 3'-hydrogen, as it is the most available hydrogen atom in the major groove followed by strand scission. [37][49]

**1.5 Significance of the Investigation of the C5'-dideoxy radical generated by LEE-DNA interaction.**

It has been well established fact that LEE effectively induces formation of TNI within the DNA subunits which further decay via Dissociative Electron Attachment (DEA) leading to formation of single as well as double strand breaks. This was supported by the study carried out to investigate the cleavage of phosphodiester backbone of DNA on exposure to LEE. Mechanism by which LEE induces strand breaks depends upon the position at which they are localized on the DNA subunit. For instance, LEE can initially
localize on the nucleobase from which it can transfer onto the phosphate group via resonance electron transfer to form a TNI at phosphate group. Scheme shows the proposed mechanism for LEE induced strand break by cleavage of the phosphodiester bond. LEE are believed to initially localize in the \( \pi^* \) orbital of the nucleobases in DNA followed by their transfer to the \( \pi^* \) orbital of the phosphate group thus forming TNI at that group. As represented in Scheme 1-13, the transient negative ion (TNI) being unstable, can decay via two possible pathways: Path A and Path B. Both these pathways involve homolytic cleavage at the C3' and/or C5' positions along with formation of different carbon centered radicals. Path A involves homolytic cleavage of the C-O \( \sigma \) bond while Path B involves homolytic cleavage of the P-O \( \sigma \) bond. Path A is the major pathway followed and it can lead to formation of C3'-dideoxy radical 64 and C5'-dideoxy radical 69 along with formation of phosphate termini from the 3' and 5' cleavage respectively. These radicals seem to be the major products associated with the cleavage of phosphodiester bonds hence it is necessary to study their chemistry. In order to understand the fate of these carbon centered radicals generated at the positions, it is necessary to generate these radicals experimentally. Formation of radical 64 and radical 69 has been observed using electron spin resonance (ESR) studies. [49], [50]
Scheme 1-13: Proposed LEE-induced cleavage of the phosphodiester bond at 3' and 5' positions of DNA. Reprinted with permission.\textsuperscript{[50]}

Study of these radical intermediates can contribute towards elucidation of the different DNA lesions and establish the mechanism of DNA damage.\textsuperscript{[50]}

1.6 Independent Generation of the Deoxyribosyl Radical

The chemical and biological impact of LEE in DNA has been studied extensively. However, interpretation and analysis of DNA damage by IR can be complicated as it can
generate a broad spectrum of reactive intermediates during the process. LEE can form series of nucleobase and sugar radicals via H-abstraction at different C-centers in the DNA biomolecule. In order to investigate the mechanism of LEE induced DNA damage, different radical precursors have been synthesized that can independently generate desired reactive intermediates at a specific site in DNA. This strategy can help in elucidation of different reaction mechanisms involved and reveal reaction pathways, thus contributing towards understanding the various biochemical events taking place. \[51\] Independent and site selective generation of the desired radical species can be achieved using different photolabile groups such as phenylselenyl or acyl groups in nucleosides as well as nucleotides. These groups can be effectively photocleaved to generate nucleobase radicals as well as sugar radicals, at different positions in a monomer and an oligonucleotide. Acyl radical precursors, undergo Norrish Type I photocleavage, during which photo-activation of various groups (isopropyl, methyl, acetyl, or pivaloyl) results in bond scission at either side of the carbonyl group. \[51\][52]

**Scheme 1-14** shows the Norrish Type I photolysis of acyl derivatives of oligonucleotides 72, which can result in bond scission via two pathways depending upon the cleavage site on either side of the carbonyl bond. In Path A, a homolytic cleavage occurs between the C-CO bond of the ketone and carbon atom of the 2-deoxyribose moiety resulting in the formation of two fragments; sugar radical 73 and acyl radical (RCO•, 74). The resulting acyl radical further undergoes decarbonylation to release carbon monoxide along with a second alkyl radical (R•). In path B, the homolytic cleavage of R-CO bond gives acyl sugar radical 75 along with an alkyl radical (R•). Compound 75 further undergoes decarbonylation to release carbon monoxide and sugar radical 73. \[52\]
R= Methyl, isopropyl or tert-butyl.

**Scheme1-14:** Norrish Type I photocleavage of radical precursor containing acyl groups.

[52]

1.7 **Significance of using Biomarkers in quantifying oxidative DNA damage**

The World Health Organization has defined a biomarker as any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease [53]. Oxidative stress to DNA leads to various mutations which can be related with pathogenesis of various inflammatory diseases and cancer. A large number of experiments have been carried out to develop biomarkers to measure the extent and nature of oxidative stress in these diseases.
A biomarker to be clinically relevant, it should meet properties like:

Criteria for Biomarker

- Can be quantified
- Posses specificity
- Has Prognostic values
- Corelate with disease pathogenesis
- Stability

Most of the studies used to quantify the oxidative damage uses reactive oxygen species (ROS)-induced modifications as biomarkers. Sample preparation for biomarker elucidation is a critical step, any spurious oxidation from other sources can give misleading results. Various sample preparation methods have been investigated, it may involve DNA extraction, derivatization before the GLC–MS analysis, digestion with enzymes, etc. Following figure shows different assay that can be used to quantify a biomarker molecule which is formed as a result of oxidative DNA damage. [53] [54]
8-oxodG (8-oxo-7,8-dihydro-2’-deoxyguanosine), 8-oxoGua (8-oxo-7,8-dihydroguanine) are believed to be the two predominant forms of free radical-induced oxidative lesions and have been used as potential biomarkers. Levels of 8-oxodG and 8-oxoGua in urine has been effectively measured and analyzed. 8-OHdG has been quantified using DNA hydrolysis [55] HPLC and Comet Assay have been used to quantify the levels of 8-oxoGua, in case of HPLC, the levels were detected in the lymphocytes while in the Comet assay, endonucleases was used for detection. [52] [54]

All these different available assays provide a promising pathway for use of different oxidized products of DNA damage as potential biomarkers and their application in clinical diagnosis of inflammatory diseases and cancer.
Chapter 2

Result and Discussion

2.1 Synthesis and photolysis of a radical precursor resulting in the formation of the 5'-deoxy- C5'-thymidinyl radical.

2.1.1 Previous studies done on synthesis of radical precursor for studying 2-deoxyribose sugar radicals

Various endogenous and exogenous sources cause free radical-induced DNA damage in living organisms by a variety of mechanisms. LEE from ionizing radiation represents a source of exogenous DNA damage. Oxidative damage of the 2-deoxyribose moiety in DNA produces a series of reactive intermediates and plays a critical role in determining their effect on the progression of diseases like cancer and other chronic inflammatory diseases. Damage to 2-deoxyribonucleotides in DNA can involve SSBs and DSBs lesions, protein–DNA cross-links, and protein and DNA adducts. The formation of C-centered radicals at different positions of the sugar moiety are key intermediates in the direct, as well as, indirect damage caused to the DNA molecule. Work has been done in the Bryant Friedrich group to investigate the generation of radical at the C3’- position of thymidine by designing a C3’-acyl nucleoside radical precursors molecule. As seen in Scheme 2-1, this work involved the effective generation of C3’ radical (78) via Norrish
type I photocleavage from both erythro- and threo-C3'-acyl substituted 2'-deoxynucleosides (76 a-c and 77 a-c). The relationship between the diastereoselectivity of the C3' radical photolysis product and the stereochemistry of the starting nucleoside was investigated. It was observed that the stereochemistry at the 3'C position of the starting ketone did not affect the photocleavage process or the diastereoselectivity of the reduction thus implying that the reduction takes place via a free radical process. [38]

Scheme 2-1: Site specific radical generation at C3' using radical precursors.
The above mentioned work is an ideal example for photolytic generation of C-centered radical for 2-deoxyribose sugar in DNA. A research group from the University of Athens, Greece, carried out the independent generation of C5'-nucleosidyl radicals from the photolysis of the C5'-tert-butyl ketone of thymidine. Scheme 2-2 depicts a model system for site-specific generation of the C5'-radical from the C5'-tert-butyl ketone derivatives of thymidine (80 and 81) which upon photoactivation, produce the thymidin-5'-yl radical (6). In the presence of a hydrogen atom donor like glutathione, the thymidin-5'-yl radical gets efficiently reduced. The C5' radical formed is on the secondary C-atom and in the absence of O2, this C5' radical can undergo reduction by hydrogen abstraction from glutathione.

**Scheme 2-2.** Formation of C5' radical from 5'-tert-butyl ketone derivatives of thymidine.

On similar lines, Greenberg and his co-workers designed a radical precursor for the C1' position of a 2-deoxyribose nucleotide the had a pivaloyl group which after photolysis generated reactive intermediates via hydrogen atom abstraction at the C1'-position.

### 2.1.2 Current Research

As described in previously illustrated examples, site selective formation of different C-centered radicals of a nucleobase or 2-deoxyribose moiety; can be achieved through
photolysis and can be an effective means to study mechanisms involved in oxidative DNA damage, as well as, the metabolic fates of lesions. Norrish Type I photochemical reactions have been successfully employed for the independent generation of such DNA radicals in a site-specific and controlled manner. This approach has been effectively used to generate various radical intermediates at different positions at the monomer and polymer levels. Current research investigated this phenomenon by the formation and analysis of a 5'-deoxy-C5'-thymidinyl radical precursor. Photolytic cleavage of the photolabile group introduced at C5' helped us determine the mechanistic fate of the 5'-deoxy-C5'-thymidinyl radical under anaerobic conditions. Photolytic cleavage of a group introduced at the C5' site will help us in observing the free radical formed at that position which can further be analyzed for its potential to act as a biomarker in DNA damage.

Scheme 2-3: Photochemical generation of 5'-deoxy-C5'-thymidinyl radical.

2.2 Design and Synthesis of a Photochemical Precursor for the 5'-deoxy-C5'-thymidinyl radical.

The most challenging step of designing efficient photoactive or photolabile radical precursors, is to pick the right acyl group at the desired position for radical formation.
Methyl ketones have been used previously as radical precursors in the generation of nucleoside-based radical intermediates, one such example was illustrated in the previously discussed Scheme 2-1. The acetyl radical is expected to be more stable than the methyl radical, hence there is a high possibility that the radical precursor with the methyl group will follow the desired path of Norrish Type I cleavage after photolysis. Therefore, it was decided to synthesize a radical precursor with a methyl ketone group at the 5′-position of 2-deoxyribose. It is essential that, the photolabile group selected should be stable throughout the synthesis and should be capable of generating the desired radical at the desired C-center upon photolysis. A methyl ketone has a carbonyl moiety that absorbs light in a range that eliminates the possibility of DNA damage by other mechanisms and subsequently undergoes Norrish Type I photocleavage. The synthesis for the 5′-deoxy-C5′-thymidinyl radical precursor is represented in Scheme 2-4, Scheme 2-5 and Scheme 2-6.

Scheme 2-4: Synthesis of compound 89 yields reflect the amount obtained in our lab.
The synthesis, which started with commercially available thymidine (84) to compound 89, was achieved as per the published literature and is shown in Scheme 2-4. TBDMS bis-protected thymidine 85 was initially made by protecting the 5’OH group followed by repeating the procedure to protect the 3’OH. Later, based on work done by Wagenknecht et al., TBDMS-bis protection was carried out in a single step instead of two steps. In this new approach, thymidine, TBDMSCl and imidazole were used in the molar ratio of 1: 2.1: 4.2, respectively delivering the compound in 95% yield. Compound 85 was subjected to acid catalyzed selective deprotection using TFA: H2O in a ratio of 10:1 to give 86. This method however delivered a yield of only 52%, hence a new strategy was employed, which used 45% aq. HF as a 15% solution in acetonitrile. These conditions are severe for selective deprotection and were surmised to lead to some amount of completely deprotected thymidine along with selectively deprotected compound 86. Therefore, the procedure was slightly modified where the solvent was changed from ACN to DCM. Aqueous HF is not completely soluble in DCM, and so the compound wasn’t exposed to high concentrations of HF. The reaction took 40 hours to reach completion and the yield obtained was 50%. The TFA/H2O deprotection method used previously took 5-7 hours and gave 52% yield and required silica gel column chromatography for purification after workup. However, the product obtained after the work up in case of HF deprotection gave 86 in pure form thus needing no further purification. The TFA approach is less time consuming and gives better yield, also it is safer than using HF.
Scheme 2-5: Selective deprotection of 85 using aq. HF.

It was noticed that the yield of product obtained from selective deprotection was highly influenced by the dryness of starting material 85. A highly dry TBDMS-bis-protected thymidine (85) gave better yield and less amount of free Thymidine. Any presence of solvent in the starting compound 85 will give a misleading mass which may lead to miscalculation of the amount of aq. HF needed. Addition of the acid in concentrations more than necessary may trigger some amount of complete deprotection along with the desired selective deprotection. Compound 87 was prepared using Dess Martin periodinane which was synthesized in our lab using a reaction scheme by Robert E. Ireland and Longbin Liu[60]. Dess Martin oxidation has been proved to be very useful for selective and mild oxidation of alcohols, therefore it was the best approach for our reactions. Compound 87 which has a 5′-aldehyde group, was isolated using non-aqueous work up to avoid loss of the compound in the form of a hydrate, thus giving an improved yield of 72% vs 55% obtained from an aqueous workup.

Compound 87 was then subjected to a Wittig reaction [57] to give 5′-alkene compound 88 in 61% yield. The Wittig Reaction can effectively generate an alkene by the reaction between an aldehyde or a ketone and a phosphonium ylide. The phosphonium salt used in our experiment gives a stabilized ylide that produces (E)-alkenes. The base used for ylide
synthesis was 2M NaHMDS (Sodium bis(trimethylsilyl) amide) in anhydrous THF. It is a strong base and has been extensively used in preparing Wittig reagents.

Compound **88** was subjected to hydroboration-oxidation using BH$_3$.oxathiane solution in THF to afford primary alcohol **89** in 45% yield. Hydroboration is the electrophilic addition of boron to an alkene, the addition is *syn* and occurs with anti-Markovnikov regioselectivity. As the alkene in question is a terminal alkene and anti-Markovnikov selectivity is desired, hydroboration becomes an ideal choice of reaction. The yield of compound **89** obtained was lower than that mentioned in the published literature (61%)$^{[58]}$. This is possibly because of the fact that 1,4-oxathiane used in the reaction was not stored under desired conditions. It is also not possible to buy this reagent as it is no longer commercially available. Since the yield was low, an alternative synthesis was tried, using 9-borabicyclo(3.3.1)nonane or 9-BBN which is an organoborane compound with greater stability than borane 1,4-oxathiane.$^{[61]}$ This approach however gave a poor yield of 30%. This decreased yield could be attributed to less selectivity of 9-BBN in comparison with BH$_3$.oxathiane for terminal alkenes. With 9-BBN, there is possible addition at the C-C double bond on the thymine base along with addition at the terminal alkene, therefore reducing the overall yield of the desired alcohol. The alkene in question is at a terminal position, therefore it was decided to continue with BH$_3$.oxathiane reagent. The synthesis of compound **92** from Compound **89** is shown in **Scheme 2-6**. Compound **89** obtained from hydroboration was further oxidized to compound **90** using Dess Martin periodinane in 83% yield.

Compound **90** was then methylated using Grignard reagent, i.e. methyl magnesium
bromide at -78 °C which afforded compound 91 in 63% yield. Use of Grignard reagent can successfully generate carbon-carbon bonds with the carbonyl group of the aldehyde, accompanied with the formation of a secondary alcohol. The alkylation yielded compound 91 as a diastereomeric mixture that was used for the next step involving oxidation of 91 using Dess Martin periodinane to afford 5'-deoxy-5'-thymidinyl radical precursor, 92 in 92% yield. Since compounds 91 and 92 are novel, they were characterized by using 1H-NMR and 13C-NMR. Spectral data is made available in Appendix B, figure 13, Figure 14 and figure 15, figure 16 for the 1H-NMR and 13C-NMR of 91 and 92 respectively. A high resolution MS was carried out for 91 as well 92, the data for which has been included in Appendix A, figure 1 and figure 2 respectively.

Scheme 2-6: Synthesis of radical precursor 92, yields reflect the amount obtained in our lab.

The previous approach to synthesize compound 92, which is covered in Scheme 2-3 and Scheme 2-6, is lengthy, involving a total of eight steps. Therefore, another line of synthesis was adapted. The new synthetic approach to obtain compound 91 has been represented in Scheme 2-7. The reason to adapt a new method was to reduce the number of steps involved and to try and improve yields for the intermediate steps. Scheme 2-7 goes
through a total of six steps, thus has the advantage of saving two steps and making the new synthesis time efficient.

Scheme 2-7: Alternative synthetic approach to make compound 92. \(^{[58]} [59] [61] [62]\) yields reflect the amount obtained in our lab.

The conversion of compound 84 to 87 follows the same synthetic route as in Scheme 2-4. In Scheme 2-7, the Wittig reaction is modified, ethyltriphenylphosphonium bromide is used instead of methyltriphenylphosphonium bromide. Compound 93 was produced in 41% yield; the lower yield can be attributed to lower E/Z selectivity due to the use of ethyl triphenylphosphonium bromide. Compound 93 is a novel compound, and the \(^1\)H-NMR and
$^{13}$C-NMR data has been included in Appendix B, Figure 17 and Figure 18, respectively. A high resolution MS carried out for 93 has been included in Appendix A, Figure 7. Product 93 was subjected to hydroboration to give a mixture of compound 91 and 94 which are regioisomers as the hydroxyl group adds to either side of the double bond of the unsymmetrical alkene (Scheme 2-8). The molar ratio in which 91 and 94 were obtained was 2:1. 5′-carbon atom of alkene is sterically hindered due to the adjacent base present, compared to the to the other carbon atom of the alkene. This could be the reason for formation of 94 in lesser yield than 91. Nevertheless, this lowered the overall yield for compound 91 to 35%. The alkene is not a terminal alkene and is unsymmetrical in nature with equally substituted carbon atoms, therefore anti-Markovnikov addition is not essential. In spite of this, we still preferred hydroboration for hydration over using acid catalyzed hydration. In presence of acid, there is a possible TBDMS deprotection that can happen which is undesirable.

**Scheme 2-8:** Formation of regioisomers 91 and 94 from hydroboration of compound 93.
2.3 Photochemical Generation of the 5'-deoxy-C5'-thymidinyl radical in the presence of tri-nButyltin Hydride under Anaerobic conditions.

Initial photolysis experiments were carried out in order to determine the ability of 5'-deoxy-C5'-thymidinyl radical precursor 92 to generate the 5'-deoxy-C5'-thymidinyl radical 95 under anaerobic conditions. As a proof of concept, the TBDMS group at 3′C was not deprotected in the radical precursor during the initial photolysis studies, in order to eliminate possible side reactions which may happen between the methyl ketone group and the 3′-OH.

Scheme 2-9: Generation of 5'-deoxy-C5'-thymidinyl radical 95.

The effectiveness of compound 92 as a radical precursor was determined through trapping of 95 with a hydrogen atom donor, tri-nbutyltin hydride. The radical precursor should undergo a Norrish Type I photochemical reaction, which involves α-cleavage of the ketone on either side of carbonyl carbon to fragment into two free radical intermediates. The C=O group accepts a photon, electrons gets excited to a photochemical singlet state, followed by entering into a triplet state via intersystem crossing. In the case of the 5'-deoxy-C5'-thymidinyl radical precursor, two different photolysis paths are possible, Path A and Path B, depending on the position of α-cleavage as represented in Scheme 2-10.
Scheme 2-10: Photolytic cleavage pathways possible for radical precursor 92.

In order to obtain the 5'-deoxy-C5'-thymidinyl radical 95, the desired path of photolysis will be Path B. In case of Path A, the resultant radical will be 96 which can either get reduced to 90 or can undergo decarbonylation to give radical 95.

Scheme 2-11: Photolysis of 92 in presence of excess tri-nbutyltin hydride at 15 °C under anaerobic conditions.

Photolysis of 92 (200 µL of 0.3mM solution) was performed in presence of argon at 15 °C using a 500 W high pressure mercury arc lamp fitted with an IR filter, focusing lens,
and 320 nm cutoff filter. The solution to be photolyzed was prepared in 1:1 acetonitrile/water containing excess tri-nbutyltin hydride which is a hydrogen atom donor. The photolysate was analyzed directly without workup using reverse phase high performance liquid chromatography (HPLC) employing a C18 column with detection wavelength at 254 nm.

**Figure 2-1**: Progress of photolysis detected by HPLC
The chromatographic monitoring of the photolysis included in Figure 2-1, clearly indicates a decrease in peak height of the starting material, compound 92 and formation of a new peak around 2.6-2.7 mins.

Appearance of a new peak in the chromatogram indicates that the 5'-deoxy-5'-thymidinyl radical precursor has undergone photocleavage to give a new product or products.

The products were isolated and identified by electrospray ionization mass spectrometry (ESI-MS). The mass observed was found to be a sodium adduct of molecular ion, MS data obtained is included in Table 2-1. The mass observed in the photolyzed sample indicates the loss of the tert-butyl group from TBDMS to give m/z corresponding to compound 98 instead of compound 97. According to the published literature, there is an evidence for the loss of tert-butyl group during the ESI-MS analysis.\(^{[63]}\)

<table>
<thead>
<tr>
<th>Observed m/z for photolyzed sample</th>
<th>Predicted possible structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated ([M + Na]^+ = 307.12)</td>
<td><img src="98.png" alt="Predicted structure" /></td>
</tr>
<tr>
<td>Observed ([M + Na]^+ = 307.4)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2-1**: Observed m/z of the photolyzed analyte in presence of a hydrogen atom donor under anaerobic conditions.
Figure 2-2: Offline ESI-MS analysis of photosylate from the photolysis of 92 in presence of excess tri-\textit{n}-butyltin hydride in 1:1 acetonitrile/water for 75 mins at 15 °C under anaerobic conditions.

The possible products if the radical precursor 95 undergoes Norrish Type II photolytic cleavage has been depicted in Scheme 2-12 along with m/z values of each product. The MS of photosylates obtained from photolysis of 92 under anaerobic conditions did not show any m/z peak corresponding to any of the Norrish Type II photolysis products.

Scheme 2-12: Possible products of Norrish Type II reaction.
Norrish Type II photocleavage requires a $\gamma$-H atom which though present in our radical precursor does not lie in the same plane as that of the carbonyl group. Thus, the possibility of abstraction of this $\gamma$-H by carbonyl carbon radical does not seem feasible, the possibility of Norrish Type II photocleavage was eliminated. Therefore, it can be concluded that, the C5'-thymidinyl radical precursor followed Norrish Type I photocleavage which falls in accordance with our requirement. The following set of figures from Figure 2-3 to Figure 2-5 depict the progress of the photolysis reaction at different time interval as indicated by reverse phase HPLC analysis.

**Figure 2-3:** Reversed-phase HPLC chromatogram of unphotolysed 92 (60 nmoles) in 1:1 acetonitrile/water in the presence of excess tri-nbutyltin hydride.
Figure 2-4: Reversed-phase HPLC chromatogram of the photolyzed 92 (60 nmoles) in 1:1 acetonitrile/water in the presence of excess tri-\textit{n}butyltin hydride after 30 mins of photolysis.

Figure 2-5: Reversed-phase HPLC chromatogram of the photolyzed 92 (60 nmoles) in 1:1 acetonitrile/water in the presence of excess tri-\textit{n}butyltin hydride after 75 mins of photolysis.
**Figure 2-5** indicated that after 75mins duration of photolysis, the starting material is almost 100 percent consumed. The possibility of Norrish Type I photolysis by path A to give compound 90 in presence of a hydorgen atom donor, was eliminated by spiking the photolysate with independently synthesized compound 90. The chromatogram seen in **Figure 2-6** indicates that compound 90 and the product obtained from photolysis are seen at different retention times, thus eliminating path A of photolysis.

![Reversed-phase HPLC chromatogram](image)

**Figure 2-6:** Reversed-phase HPLC chromatogram of photolyzed 92 (60 nmoles) in 1:1 acetonitrile/water spiked with compound 90 in the presence of excess tri-nbutyltin hydride after 30 mins of photolysis.

### 2.4 Photochemical Generation of the 5'-deoxy-C5'-thymidinyl radical in the absence of tri-nbutyltin Hydride under Anaerobic conditions.

Photolysis of 92 (200 µL of 0.3mM solution) was performed using the same method
as described above except no hydrogen atom donor was added. The crude photolysate was injected into HPLC without any workup and the products obtained were detected at 254 nm. The analysis of the crude photolysate indicated the formation of the radical disproportionation products. Radical disproportionation is a radical reaction in which two radicals react within each other to form two new non-radical products. Because of the unstable nature of radicals, disproportionation phenomenon proceeds rapidly with little to no activation energy necessary. The phenomenon of disproportionation leads to formation of mainly 102 while 97 is only formed in traces when photolysis was carried out in the absence of a hydrogen atom donor. **Scheme 2-13** demonstrates the disproportionation mechanism which involves H-atom abstraction by the acetyl at the C5’ thymidinyl radical.

**Scheme 2-13**: Disproportionation between the C5’-thymidinyl radical 95 and acyl radical, a proposed mechanism for formation of unsaturated product 102 in the absence of a hydrogen atom donor.

The MS analysis of the photolyzed sample in this case also showed loss of tert-butyl group hence the mass observed was for compound 103 instead of compound 102 as shown in **Table 2-2** below.
<table>
<thead>
<tr>
<th>Observed m/z for photolyzed sample</th>
<th>Predicted possible structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated [M + Na]$^+$ = 305.1</td>
<td><img src="image" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>Observed [M + Na]$^+$ = 305.3</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2-2:** Observed m/z of the photolyzed analyte in absence of a hydrogen atom donor under anaerobic conditions.

**Figure 2-7:** Offline ESI-MS analysis of photosylate from the photolysis of 92 in the absence of excess tri-\(n\)-butyltin hydride in 1:1 acetonitrile/water for 75 mins at 15 °C under anaerobic conditions.
Thus from the analysis of photolysis products, in the presence as well as absence of a hydrogen atom donor, it is clear that radical precursor 92 gave the desired 5'-deoxy-C5'-thymidinyl radical 93. It was decided to remove the TBDMS group from the 3'C to give compound 104, photolysis studies on compound 104 would be physiologically relevant than compound 92. Deprotection of TBDMS group was carried out by using 2M TBAF in THF at 0 °C.

Scheme 2-14: Removal of TBDMS group from compound 92

The deprotection was achieved but purification to separate product 104 from TBAF was challenging. Since the amount of compound 104 to be purified was in the range of mg, a pipette was used instead of a standard column for chromatographic separation. In spite of good separation and a good MS for the purified fraction, the compound was not pure by NMR. The reason for this could be the possible cyclization of compound 104. In 104, the hydroxyl group is four carbons away from the carbonyl carbon thus the possibility of nucleophilic attack on carbonyl carbon involving ring closure is possible. If Baldwin’s rule of cyclization is applied to the current situation, there is a high possibility of a ring closing reaction by 5-exo-trig, since the carbonyl atom which is being attacked is sp2 hybridized.
Scheme 2-15: Example to illustrate Baldwin’s rule of cyclization.

As per Baldwin’s rule, 5-exo-trig is a stable ring structure and the fact that in compound 104, the position of the oxygen atom in the OH with respect to that of the carbonyl carbon is favorable to provide an optimum length for the linking chain to achieve the required trajectory for ring formation.

Scheme 2-16: Cyclization proposed for compound 104.

Both compounds have the same mass so it is not possible to distinguish them using MS. Although studies on compound 104 will be more relevant physiologically than compound 92, they could not be carried out due to the cyclization product obtained. MS for compound 104 is in Appendix A, Figure 8.
Chapter 3

Result and Discussion

3.1 Synthesis and study of small molecule degradation products formed from the 3’-oxonucleotide lesion

Damage to the 2-deoxyribose sugar in DNA leads to formation of dozens of mutagenic and cytotoxic products which can be formed due to various chemical mechanisms, including oxidation, halogenation, alkylation, etc. These products are site-specific and each of the five different C-centers of the sugar moiety has a typical spectrum of degradation products obtained. For example, oxidation of the 3’-radical, followed by solvolysis by H$_2$O gives a 3’-oxo-nucleotide residue which undergoes β-/δ-eliminations to release 2-methylene-3(2H)-furanone. There is a growing interest in employing these DNA damage products as biomarkers of disease as there is a strong association between DNA damage and disease pathology has been established. [58] To exploit the small molecules of DNA products as biomarkers, it is essential to have a thorough knowledge with respect to mechanistic information regarding the fates of the damage products in terms of metabolism and distribution.

These biomarkers represent a promising future to quantify the severity of the inflammatory
processes to determine the level of oxidative stress in a disease. We have attempted to study the degradation product formed from the 3′-oxo-nucleotide lesion with a special focus on 2-methylene-3(2H)-furanone. In this process, we have carried out the site specific generation of deoxyribonolactone sites within 3′-oxo-thymidine nucleoside and follow the different possible products obtained.

Approaches employed to generate small molecule degradation products from 5′-O-(tert-butylidimethylsilyl)-2′-deoxythymidine-3′-one (109) has been explained below.

![Scheme 3-1: Synthesis of compound 109 from thymidine](image)

Commercially available thymidine 84 was protected at 5′ position using TBDMSI to give compound 108, which was further oxidized using Dess Martin Periodinane to give 5′-O-(tert-butylidimethylsilyl)-2′-deoxythymidine-3′-one 109 in 99% yield. The next step was to remove the 5′-TBDMS group for which different approaches were used. Removal of the 5′-TBDMS imparts instability to the resultant compound which therefore facilitated spontaneous degradation to give a series of small compounds that were observed using ESI-MS and NMR. Various ways to deprotect the 5′-TBDMS group exists, which includes several methods under acidic conditions and other under basic conditions. The most commonly used technique for TBDMS removal is to use base deprotection using TBAF (Tetra-n-butylammonium fluoride) which was attempted in the beginning but
interpretation of the products formed by this procedure was obscure and complicated, therefore it was decided to proceed using acidic deprotection.

3.1.1 Aq. HF solution for the removal of TBDMS group.

Scheme 3-2: Proposed fate of 109 in presence of aq. HF

The reaction with aqueous HF moved fast and reached completion in 45 mins. The TBDMS group at the 5' position was effectively removed to give compound 110. The product 110 obtained was analyzed using $^1$H-NMR and $^{13}$C-NMR that is included in Appendix B (Figure 19 and Figure 20, respectively), and MS result are shown in Figure 9 in Appendix A. Compound 110 was found to degrade into compound 111 and free thymine 112, if the reaction was carried out for longer period (about 3 hours), or was subjected to purification using column chromatography.

3.1.2 TFA solution for the removal of TBDMS group.

Scheme 3-3: Proposed fate of 109 in presence of 2% TFA in DCM.
After employing an inorganic acid like HF, it was decided to try deprotection using an organic acid. Since TFA (Trifluoroacetic acid) has been previously used for TBDMS deprotection, it was selected for the reaction. The reaction was then carried out in the presence of 2% TFA in DCM as per published literature \[^{66}\] to obtain compound 110. However, this reaction was allowed to run overnight in order to study the possible degradation products. After about 30 hours, the reaction product mixture showed the expected compound 110 along with two degradation products, compound 113 and free thymine 112. The relevant MS data has been included in Appendix A, Figure 10. From the above experimentation, it was clear that inorganic acid offers a promising path to obtain 2-methylene-3(2H)-furanone since HF delivered efficient TBDMS deprotection in comparison to TFA.

### 3.1.3 HCl solution for the removal of TBDMS group.

Since aqueous HF is not safe to handle, similar experiments were carried out using 5% HCl in EtOH. The reaction reached completion in 3 hrs, and the product mixture obtained was found to contain compound 111 along with free thymine 112, thus indicating TBDMS deprotection as well as base elimination. After purification, different fractions where analyzed by ESI-MS and \(^1\)H-NMR in Appendix A, Figure 11 and Appendix B, Figure 21, respectively. One of the fractions obtained also showed a m/z peak which may correspond to 2-methylene-3(2H)-furanone (31). However due to the instability of 31, it could not be characterized using \(^1\)H-NMR.
An efficient method to isolate or couple 2-methylene-3(2H)-furanone has to be devised. T. P. Begley et al have been successful in-situ generation of 2-methylene-3(2H)furanone by the addition of triethylamine to of 3-keto-5-tosylthymidine. \[^{[67]}\] This approach was adapted in our studies as well, where the fractions obtained were treated with an excess of 3-Mercaptobenzoic acid, this proved as a mean to test presence of 2-methylene-3(2H)-furanone 31. The aromatic thiol coupled with 2-methylene-3(2H)furanone 31, the mass spectrum for the coupled product is shown in Appendix A, Figure 12.
Chapter 4

Conclusion and Future Research

4.1 Conclusion

5'-deoxy-C5' -thymidinyl radical precursors 92 was successfully synthesized by employing two organic synthetic pathways. In order to understand the mechanisms involved in LEE induced damage to DNA, the fate of 92 was determined through its independent generation and elucidation of the structures resulting from photolysis, thus giving an idea of the final DNA damage products. The efficiency of photochemical generation of 5'-deoxy-C5'-thymidinyl radical 95 from 92, was investigated under anaerobic conditions in the presence, as well as absence of excess tri-nbutyltin hydride which is a hydrogen atom donor. The formation of reduction product 97 (detected as compound 98) confirms the suitability of 3'-O-(tert-Butyldimethylsilyl)-5'-acetyl-5'- deoxythymidine, 92 as a precursor for 3'-O-(tert-butyldimethylsilyl)-5'-acetyl-5'- deoxythymidinyl radical 95. Compound 104 could not be exposed to photolytic cleavage because of its instability and tendency to undergo ring closure.

With respect to the other project involving formation of 2-methylene-3(2H)-furanone from 3'-oxo-nucleotide, use of aq. HF seems to be an optimistic approach to get compound 110
in comparison to use of TFA, however, the safety of using HF on large scale could be an issue. The approach adapted by using aq. HCl seems to work well, but it is essential to establish the reproducibility of the process to affirm the findings.

4.2 Future Research

To facilitate this investigation in the future, different photolabile acyl groups can be site-specifically developed in order to compare the efficiency of these groups to form the desired radical. The work explained in Table 4-1 was carried out by Bryant Friedrich lab, wherein various acyl groups where investigated to find the best possible side chain for the ketone radical precursor.\textsuperscript{[37]}

![Figure 4-1: Structures of ketone radical precursor designed to synthesize the C3’-thymidinyl radical.](image)

Analysis of photolyzed products for ketones76 a-c and 77 a-c indicated that, tert-butyl ketones 76b and 77b underwent an effective Norrish type I Cleavage giving the highest yields with minimum side products. Methyl ketone 77 a-c was found to be a good choice too, but the yield was less than the latter as it may have likely produced more number of side products. The third group which was tried was benzoyl ketones 76c and 77c which gave the lowest yields.
<table>
<thead>
<tr>
<th>Groups attached</th>
<th>Length of Irradiation</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R= CH₃ (76a)</td>
<td>3h</td>
<td>20</td>
</tr>
<tr>
<td>R= CH₃ (77a)</td>
<td>3h</td>
<td>65</td>
</tr>
<tr>
<td>R= (CH₃)₃C (76b)</td>
<td>1h</td>
<td>79</td>
</tr>
<tr>
<td>R= (CH₃)₃C (77b)</td>
<td>1h</td>
<td>92</td>
</tr>
<tr>
<td>R= Ph (76c)</td>
<td>2h</td>
<td>35</td>
</tr>
<tr>
<td>R= Ph (77c)</td>
<td>2h</td>
<td>33</td>
</tr>
</tbody>
</table>

**Table 4-1:** Percent yield of photolysis products of ketones 76 a-c and 77 a-c

From the above mentioned study, it can be seen that pivaloyl group works best for such photolysis experiments to prepare the radical precursor. Therefore, in the present study, we can synthesize such modified nucleosides containing various photolabile groups, especially the pivaloyl group. Based on the results from photolysis data from these other groups, a conclusion can be reached about the yield of desired radical at 5'C of deoxyribose sugar moiety. The group which works best can be used to selectively generate the reactive intermediate of interest in an oligonucleotide strand in a controlled manner.

With respect to the other research project that involved study of small molecule degradation product from, only preliminary data has been obtained. The different approaches used to
obtain the degradation products must be optimized for their reproducibility and to verify the findings. Once 2-methylene-3(2H)-furanone is successfully isolated, its reactivity and fate can be investigated in the presence of several nucleophiles.
Chapter 5

Experimental Procedures

All experiments were performed with standard laboratory instruments, reactions were carried out under an argon atmosphere, oven-dried and clean glassware were used, extra care was taken in the procedures demanding water and oxygen exclusion. All chemicals used in synthesis were handled and used as per the respective standard operating procedures (SOPs).

5.1 Materials

All chemicals, reagents and solvents were purchased from the following list of commercial suppliers: Acros Organics, Pharmaco-AAPER, Chem-Impex, Sigma-Aldrich, and Fisher Scientific. Deuterated solvents for NMR (Deuterated Chloroform and Deuterated DMSO) were purchased from Cambridge Isotope Laboratories. For chromatographic separation using column chromatography, analytical grade solvents were used from Sigma. For separations using HPLC, HPLC grade solvents where used. Deionized water needed for HPLC analysis was obtained from PURELAB® Ultra Water Purification System. Triethylammonium acetate buffer (1M) was bought from Calbiochem. The Dess-Martin reagent was synthesized in lab. [60]
5.2 Structural Analysis

All synthesized products were characterized by NMR spectroscopy and mass spectrometry, low resolution MS while high resolution MS was done for novel compound.

5.2.1 NMR Spectroscopy

5.2.1.1 $^1$H-NMR

All $^1$H-NMR spectra were acquired on either of the three different instrumentations available: a Varian VXR-400, Varian Unity Inova-600 NMR, or Bruker-Avance III-600 Nuclear Magnetic Resonance (NMR) spectrometer. All NMR samples were prepared in deuterated CDCl$_3$ or DMSO. Chemical shifts are reported in parts per million, coupling constants (J) are reported in hertz (Hz). Multiplicity is represented as: s = singlet, d = doublet, dd = doublet of doublet, t = triplet, dt = doublet of triplet, q = quartet, m = multiplet. Since the nucleotide used was thymidine, its different protons attached to the carbon atoms and nitrogen atom are represented in Figure 5-1.

![Figure 5-1: Assignment of protons in thymidine](image)

5.2.1.2 $^{13}$C-NMR

$^{13}$C-NMRs for the all the intermediates of C5'-thymidinyl radical precursor synthesis as well as for the C5'-thymidinyl radical precursor were performed on a Bruker-Avance
III-600 NMR spectrometer in CDCl₃, the triplet at δ 77.23 for CDCl₃ was used as an internal standard. The $^{13}$C-NMRs are $^1$H broadband-decoupled.

5.2.2 Mass Spectrometry

5.2.2.1 ESI-MS

Mass spectrometry was performed on an Esquire electrospray ionization (ESI) mass spectrometer (Bruker Daltonics, Bremen, Germany) operated in the positive ion mode and equipped with a quadrupole ion-trap mass analyzer. The samples were dissolved in methanol to make final concentration of 0.1-0.5 µg/mL. The signals obtained correspond to the [M+H]⁺ or [M+Na]⁺ ions of the analyte.

5.2.2.2 High Resolution Mass Spectrometry

High resolution mass spectrometry (HRMS) was performed on a Micromass QToF II mass spectrometer located at the Mass Spectrometry and Proteomics Facility, The Ohio State University and The University of Toledo.

5.2.3 Chromatographic Methods

5.2.3.1 Thin Layer Chromatography

Thin layer chromatography (TLC) was used to monitor progress of all organic reactions, and to monitor the elution of compounds from flash chromatography. TLC was performed using silica gel 60 F254 aluminum backed plates as well as glass backed plates. Compound spots were visualized by UV light (254 nm) and stained with p-anisaldehyde dip that was previously made using: 180 mL of absolute ethanol, 10 mL concentrated sulfuric acid, 2 mL glacial acetic acid, and a few drops of p-anisaldehyde. The TLC plates were burnt using heat gun, this facilitated the visualization of spots after exposing them to
the dip. In some cases, Potassium Permanganate dip was used which was prepared using:
3g KMnO₄, 20g K₂CO₃, 5 ml 5% NaoH (aq) and 300 ml H₂O.

5.2.3.2 Flash Chromatography

Purification was performed on a Biotage SP4 chromatography system equipped with an in-line variable wavelength detector where products were detected at 254 nm. TLC was used to determine the purity of eluents fractions, and those containing the desired pure compound were combined and evaporated under reduced pressure using a Heidolph Collegiate Brinkmann rotary evaporator. Also manual column where set up for purification of samples which were more than 5g in weight, the column was wet packed with silica flash grade G60.

5.2.3.3 HPLC

All high performance liquid chromatography (HPLC) analysis were performed on a Dionex Ultimate 3000 reverse phase HPLC system equipped with an in-line variable wavelength 93 detector. The analysis includes identification and quantification of the products. Type of reverse-phase column used:

Thermo Hypersil-Keystone BDS Hypersil C-18 column, 4.6 x 250 cm, 5 µm.

The solvent systems used were:
Solvent A: 50 mM TEAA buffer pH = 7.0.
Solvent B: Acetonitrile.

5.3 Other Equipment and Devices

High vacuum pump – Edwards RV3.
Rotary evaporator – Heidolph Collegiate Brinkmann rotary evaporator.
Stilt to obtain anhydrous THF.
5.4 Synthesis and Photolysis of Nucleoside Radical Precursor

5.4.1 Synthesis of 3'-O-(tert-Butyldimethylsilyl)-5'-acetyl-5'-deoxythymidine (92)

5.4.1.1 Synthesis of 3'-O-(tert-Butyldimethylsilyl)-5'-C-(hydroxymethyl)-5'-deoxythymidine (89)

It was synthesized as reported in published literature.\textsuperscript{[58]}

5.4.1.2 Synthesis of 3'-O-(tert-Butyldimethylsilyl)-5'-C-formyl-5'-deoxythymidine (90)

\[ \text{A solution of 89 (0.6 g, 1.617 mmol) in anhydrous CH}_2\text{Cl}_2 (6 mL) was cannulated into a solution of Dess-Martin periodinane (1.03 g, 2.425 mmol) in anhydrous CH}_2\text{Cl}_2 (13 mL) at 0 ^\circ \text{C and stirred for about 30 mins under argon atmosphere. After 30 mins, the reaction mixture was allowed to stir overnight at room temperature under inert conditions using argon. The non aqueous work up involved removal of CH}_2\text{Cl}_2 by using rotary evaporation followed by the addition of 50 ml of ethyl acetate to the reaction mixture, this was allowed to stir for 15-20 mins. The reaction mixture was then filtered by vacuum filtration through a ‘fine pore’ frit funnel with a layer of celite on the top of the funnel. Anhydrous MgSO}_4 was added to the filtrate obtained and allowed to stir overnight. Next day, the solution was filtered through a ‘fine pore’ frit funnel with a layer of cotton on the top of the funnel. The} \]
filtrate obtained was then evaporated to dryness in vacuo. The product 90 was isolated as a white foamy solid in 83% yield.

5.4.1.3 Synthesis of 1-[(3-O-tert-Butyldimethylsilyl)-5-C-(2-hydroxypropyl)-2,5-dideoxy-pentofuranosyl] thymine (91)

0.5g (1.35mmol) of 90 was dissolved in 18 ml of anhydrous diethyl ether, the solution was cooled to –78 °C followed by dropwise addition of 3 mole eq. of methy magnesium bromide, i.e; 4.05mmol, 1.35ml of 3M solution of methy magnesium bromide in diethyl ether. The reaction was allowed to run for 7 hours after which it reached completion as per the TLC. The reaction was carried out under argon atmosphere and completely anhydrous reaction environment. The reaction was quenched by adding 20 ml of DI water and 20 ml of aq. 2M HCl. This mixture was extracted twice with diethyl ether, 40 ml each. The combined organic layer was washed with 25 ml of aq. saturated NaHCO₃, dried with anhydrous MgSO₄, filtered and evaporated to dryness in vacuo to give white anhydrous solid. The reaction product was obtained as racemic mixture in 63% yield. High Resolution Mass Spec is available in Appendix A, figure 1.

¹H-NMR (CDCl₃, 600 MHz): The two isomers appeared together in the NMR, hence the protons corresponding to few peaks could be cumulative of the two isomers,
δ: 0.07 (12H, s, 6H for each isomer), 0.90 (18H, s, 9H for each isomer), 1.93 (6H, s,3H for each isomer), 1.74 (5’ 2H, m), 1.82 (5’ 2H, m), 1.93 (6H, s, 3H for each isomer), 2.14 (2H,m), 2.25 (2H, m) 3.89 (1H, m), 3.98 (1H, m), 4.05 (2H, m, 1H for each isomer), 4.12 (2H, m, 1H for each isomer), 6.16 (1H, t), 6.19 (1H, t), 7.13 (2H, s,1H for each isomer), 9.28 (2H, s,1H for each isomer)

13C (CDCl3, 600MHz) δ: -4.63, 0.18, 12.89, 14.38, 18.10, 23.26, 23.95, 25.89, 40.21, 42.07, 65.25, 67.04, 75.08, 75.31, 83.82, 85.11, 85.78, 111.38, 111.64, 135.53, 150.47, 163.97.

5.4.1.4 Synthesis of 1-[(3-O-tert-Butyldimethylsilyl)-5-C-(5-(E)-prop-1-ene)-2,5-dideoxy-pentofuranosyl] thymine. (93)

To a stirred suspension of ethyl triphenylphosphonium bromide (3.02g, 8.47 mmol) in anhydrous THF (20 ml), 5ml of 20% potassium tert-butoxide (8.47 mmol) solution in THF was added. The mixture was allowed to stir at 0 ºC for 45 mins and then at room temperature for 2 hrs under argon atmosphere. To this mixture, 1g (2.82 mmol) solution of compound 87 was added, and the reaction was allowed to run overnight, with TLC monitoring at regular intervals. The reaction workup involves addition of 120 ml of diethyl ether to the reaction mixture followed by filtration through a layer of celite placed over a ‘fine’ graded frit funnel. The filtrate was evaporated to dryness using vacuo. The crude compound obtained was purified using column chromatography using 20% ethyl acetate in CH2Cl2 to give compound 93 in 41% yield. High Resolution Mass Spec is available in Appendix A, figure 7.
$^1$H-NMR (CDCl$_3$, 400 MHz) δ:

0.09 (6H, s), 0.90 (9H, s), 1.75 (3H, d), 1.88 (3H, s), 2.18 (1H, dd), 2.23 (3H, s), 2.35 (1H, dd), 4.15 (dt), 4.67 (dd), 5.42 (m), 5.78 (dd), 6.22 (1H, t), 7.18 (1H, s)

$^{13}$C (CDCl$_3$, 600MHz) δ: 163.46, 149.99, 135.31, 130.92, 127.14, 110.86, 85.15, 82.07, 41.02, 25.68, 17.98, 13.74, 12.72, 4.86.

**5.4.1.5 Synthesis of 3’-O-(tert-Butyldimethylsilyl)-5’-acetyl-5’-deoxythymidine (92)**

(5’-deoxy-C5’-thymidinyl radical precursors)

A solution of 91 (0.3 g, 0.78 mmol) in anhydrous CH$_2$Cl$_2$ (3 mL) was cannulated into a solution of Dess-Martin periodinane (0.5 g, 1.2125 mmol) in anhydrous CH$_2$Cl$_2$ (7 ml) at 0 °C and stirred for about 30mins and then stirred overnight at room temperature under argon atmosphere. After stirring overnight at room temperature, aqueous work up was carried out by quenching with diethyl ether (30 ml), the solution was then poured slowly into a ice cold solution of saturated NaHCO$_3$(15 ml) containing anhydrous Na$_2$S$_2$O$_3$ (1.93 g). The organic layer was separated and the aqueous layer was extracted with diethyl ether (50 ml). The combined organic phase was washed with saturated NaHCO$_3$ solution (30 ml) followed by H$_2$O (25 ml), saturated NaCl solution (25 ml), dried with anhydrous Na$_2$SO$_4$ and evaporated to dryness in vacuo. The product 92 was isolated as a white foamy solid in 92 % yield.
High Resolution Mass Spec is available in Appendix A, figure 2.

$^1$H-NMR (CDCl$_3$, 400 MHz) $\delta$:
0.09 (6H, s), 0.90 (9H, s), 1.95 (3H, s), 2.23 (3H, s), 2.25 (2H, dd), 2.76 (1H, dd), 2.82 (1H, dd), 4.14 (1H, m), 4.29 (1H, m), 6.16 (1H, t), 7.19 (1H, s), 8.44 (1H, s)

$^{13}$C (CDCl$_3$, 600MHz) $\delta$: 206.12, 163.70, 150.20, 138.24, 111.38, 85.65, 82.67, 74.37, 46.28, 40.31, 30.84, 25.92, 18.14, 12.85, 0.23.

5.4.2 Photolysis Experiments

Photolysis experiments were carried out in 4x10 mm quartz cuvettes (Sigma Aldrich). Sample once prepared was exposed to a photolysis lamp which was Oriel 500W High Pressure Mercury Arc Lamp (Newport, Irvine CA) fitted with an Infra Red filter, focusing lens along with a 320 nm cut-off filter.

The photochemical reactions were performed at 15 °C temperature using an integrated Peltier PTP-1 single cell temperature controller system (Varian, Palo Alto, CA). The irradiated mixtures were analyzed directly without workup by reversed-phase HPLC with UV detection at 254 nm. HPLC analyses were carried out on C-column mentioned in section 5.3.3.3 using solvent systems comprising of ACN and TEA buffer as described in the same section. A stepwise gradient was applied. 30-45% B over 12 min, 45-55% B over 8 min. Flow rate maintained was 1.0 mL/min
5.4.3 Photochemical Generation of the 3'-O-(tert-butyldimethylsilyl)-5'-acetyl-5' -deoxythymidinyl radical from 92 under Anaerobic conditions

5.4.3.1 Photolysis of 92 in the Presence of tri- nButyltin hydride as a Hydrogen Atom Donor

A solution of radical precursor 92 (60 nmole/ 200µl) in 1:1 CH₃CN/H₂O was Taken in a quartz cuvette and degassed by bubbling argon through the solution for 20 min. tri-nButyltin hydride (100 equivalents) was added under an inert atmosphere. The mixture was immediately photolyzed for different time intervals, 30min, 45 mins, 60 mins and 90 mins at 15 °C. The irradiation product mixture was analyzed without workup and by injecting into reversed-phase HPLC system and the solvents described in the preceeding section. Products were identified by ESI-MS.

5.4.3.2 Photolysis of 92 in the Absence of tri- nButyltin hydride as a Hydrogen Atom Donor

A photolysis sample containing 92 (60 nmol,200µl) in CH₃CN/H₂O 1:1 v/v was transferred to a quartz cuvette and purged with argon for 20 minutes. The sample was immediately photolyzed for different time ranges as described in previous study at 15 °C under an inert atmosphere. The irradiation product mixture was analyzed without workup and by injecting into analytical reversed-phaseHPLC and the solvent system described in experimental section. The products were identified by ESI-MS and by spiking the photolysate with authentic samples.
5.5 Synthesis of thymidine-3′-one

5.5.1 Synthesis of thymidine-3′-one (110) using aq. HF

Dissolve 0.5g (2.8 mmol) of compound 109 in 20ml of acetonitrile, allow the solution to cool to 0°C. Add 4.4 ml of 48% aq. HF, the reaction was monitored using TLC, which indicated that the reaction reached completion in 45 mins. Two approaches where adapted for the reaction work up,

Firstly, the reaction mixture was treated with TEA (Triethylamine) in order to neutralize the HF, it led to a precipitate formation which was removed by using a cotton plug over a ‘fine pore size’ frit funnel. The filtrate was then dried under vacuum to yield the product which was analyzed using NMR and ESI-MS. This work up gave confusing NMR which could not be interpreted therefore another work up approach was tried;

In the second method, the reaction mixture was extracted using CHCl₃ (30 ml) and water (30 ml). The HF seemed to go in aqueous layer. The organic layer was then washed with saturated NaHCO₃ solution (15 ml) dried with anhydrous Na₂SO₄ and evaporated to dryness in vacuo to afford off white product of compound 110 in 32% yield.

H-NMR (DMSO, 600 MHz) δ:

1.76 (3H, s), 2.67 (1H, dd), 2.88 (1H, dd), 3.65 (2H, s), 4.09 (2H, dd), 6.38 (1H, t), 7.81 (1H, s), 11.38 (1H, s)
5.5.2 Synthesis of thymidine-3′-one (110) using ethanolic HCl

Dissolve 0.5g (2.8 mmol) of compound 109 in 20ml of 5% HCl in ethanol, at 0 °C, the reaction was monitored using TLC, which indicated that the reaction reached completion in 2hrs. Add 20 ml of DCM to the reaction mixture, and extract the organic layer with 20 ml water, followed by washes with saturated soln. of NaHCO$_3$ (20ml) and saturated NaCl soln. (20ml). Dry the organic layer with anhydrous Na$_2$SO$_4$ and evaporated to dryness in vacuo to give off-white colored compound 110 in 40% yield. When subjected to purification using column chromatography, the compound was found to degrade into products 111 and 112.

$^1$H-NMR for compound mixture of 111 and 112 (DMSO, 600 MHz) $\delta$: 
1.72 (3H, s from Thymine), 3.64 (1H, m from the 2-(hydroxymethyl)furan-3(2H)-one), 3.80 (1H, m from the 2-(hydroxymethyl)furan-3(2H)-one ), 4.54 (1H, t from the 2-(hydroxymethyl)furan-3(2H)-one ), 5.07 (1H, br m, from the 2-(hydroxymethyl)furan-3(2H)-one for –OH proton), 5.74 (1H, d, from the 2-(hydroxymethyl)furan-3(2H)-one), 7.24 (1H, s from Thymine), 8.72 (1H, d, from the 2-(hydroxymethyl)furan-3(2H)-one)

$^{13}$C (DMSO, 600MHz) $\delta$: 12.08, 40.63, 60.73, 81.30, 82.71, 109.50, 136.86, 150.42, 163.61, 210.79.
References


91


Appendix A

Mass Spectrometry data

Figure 1: High resolution ESI-MS for compound 91.
Figure 2: High resolution ESI-MS for compound 92.
**Figure 3:** Offline ESI-MS analysis of photosylate from the photolysis of 92 in presence of excess tri-*n*-butyltin hydride in 1:1 acetonitrile/water for 75 mins at 15 °C under anaerobic conditions.

**Figure 4:** Offline ESI-MS analysis of photosylate from the photolysis of 92 in absence of excess tri-*n*-butyltin hydride in 1:1 acetonitrile/water for 75 mins at 15 °C under anaerobic conditions.
Figure 5: ESI-MS analysis of compound 91 which is obtained from 90 using Scheme 2.7

Figure 6: ESI-MS analysis of compound 94 which is regioisomer of 91
Figure 7: High Resolution MS analysis of compound 93
Figure 8: ESI-MS analysis of compound 104.
Figure 9: ESI-MS analysis of 110 using 45% aq. HF.
Figure 10: ESI-MS analysis of 113 obtained from 109 using TFA
Figure 11: ESI-MS analysis of 110 obtained using 5% aq. HCl.
Figure 12: LC-MS analysis of compound 31 coupled with 3-mercaptobenzoic acid.
Appendix B

NMR data

$^1$H NMR and $^{13}$C NMR Data
Figure 13: $^1$H-NMR of compound 91 in CDCl$_3$
Figure 14: $^{13}$C-NMR of compound 91 in CDCl$_3$
Figure 15: $^1$H-NMR of compound 92 in CDCl$_3$
Figure 16: $^{13}$C-NMR of compound 92 in CDCl$_3$
Figure 17: $^1$H-NMR of compound 93 in CDCl$_3$
Figure 18: $^{13}$C-NMR of compound 93 in CDCl$_3$
Figure 19: $^1$H-NMR of compound 110 (synthesized using HF) in DMSO

Figure 20: $^{13}$C-NMR of compound 110 (synthesized using HF) in DMSO
Figure 21: $^1$H-NMR of compound mixture containing 111 and 112 in DMSO